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Studies on the Binding of Androgens to the Cytosol Proteins and Nuclei of the Rat Seminal Vesicle

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(Received March 3, 1976)

Mince of the seminal vesicles of rats castrated 5—6 days before the experiment was incubated at 37°C with ^{3}H-testosterone. After 60 min incubation with 10 nM testosterone 73% of the label bound to cytosol proteins and 83% of the label attached to nuclei was in 5α-dihydrotestosterone while unbound steroids contained only 29% dihydrotestosterone.

The amount of dihydrotestosterone bound to cytosol proteins and nuclei was 0.4 pmol/g tissue and 1 pmol/g tissue, respectively. On the basis of the nuclear binding of this steroid about 1900 binding sites per nucleus were calculated.

Both cytosolic and nuclear binding sites were half saturated at about 1 nM testosterone concentration. Most of the binding to cytosol proteins occurred during the first 10 min, while the maximum binding to nuclei was attained after 40 min of incubation.

Nuclei were found to bind considerable amounts of testosterone. Binding of testosterone to nuclei reached its maximum between 10—20 min of incubation and declined then to a comparatively low level during the ensuing 40—50 min. Various experimental results suggest that testosterone and dihydrotestosterone are accumulated in the nuclei by independent mechanisms.

Introduction

The most widely used model organ for studies of the molecular mechanisms of androgen action is the ventral prostate of the rat. Therefore, it is not surprising that most of the investigations on the binding of androgens to cytoplasmic and nuclear receptors have been carried out using the prostate of adult rats, while comparatively little knowledge has accumulated concerning the receptors of other androgen dependent tissues.

Studies in our laboratory have shown that seminal vesicle of the rat is capable of producing very high quantities of a clottable secretory protein which is responsible for the formation of the so-called "copulation plug" in this rodent

The following trivial steroid names are used: testosterone = 17β-hydroxy-4-androstene-3-one; dihydrotestosterone = 17β-hydroxy-5α-androstane-3-one; 19-nortestosterone = 17β-hydroxy-4-oestrene-3-one; androstanediol = 5α-androstane-3,17-diol; androstanedione = 5α-androstane-3,17-dione.

(Mányai, 1964a). It has also been found that this protein has a very specific amino acid composition (Mányai et al., 1965), can abundantly be prepared from the vesicular secretion (Mányai, 1964a) and can be extracted with simple procedures from the vesicular tissues (Mányai, 1964b; Tóth, Zakár, 1971). Since the biosynthesis of this protein is highly sensitive to androgens (Tóth, Mányai, 1968a), it appears as a possbile model for the study of the androgen regulation of the synthesis of a well-defined protein (Tóth, Zakár, 1971). Therefore, the mechanisms by which androgens exert their regulatory action in the seminal vesicles are of special interest.

Data available from the relatively few reports concerning the binding of androgens in this gland suggest the reductive metabolization of testosterone (Stern, Eisenfeld, 1969; Fang et al., 1969; Buric et al., 1972), the formation of a receptor 5α-dihydrotestosterone complex in the cytosol and its subsequent translocation to the nucleus (Tveter, Unhjem, 1969; Sar et al., 1970; Mainwaring, Irving, 1973). Although these processes are essentially similar to the mechanism revealed for the prostate gland (cf. King, Mainwaring, 1974), it seems highly probable that many of the details found with the prostate will not be applicable to the vesicular system without further, at least quantitative, modifications.

In order to gain more insight into the binding mechanisms of androgens in the seminal vesicles, we studied this process by incubating the mince of the vesicles *in vitro* with labelled testosterone. The characteristics of the binding are the subject of this paper.

Materials and methods

Incubation – Seminal vesicles were obtained from adult male rats (Wistar strain) killed 5 or 6 days following castration. The mince prepared from 0.8 – 1.5 g of the seminal vesicles was incubated in Krebs phosphate medium (Krebs, 1950) with ^{3}H-testosterone. The incubations were carried under continuous shaking of the reaction mixtures at 37°C in oxygen atmosphere. After incubation the reaction mixtures were cooled in an ice bath, the mince was rinsed thrice with cold physiological saline and homogenized with medium A (0.33 M sucrose, 50 mM Tris-HCl, pH 7.5, 3 mM $CaCl_2$).

Preparation of cytosol and nuclear fractions – The homogenate was filtered through 4 layers of gauze and centrifuged at 900 g_{av} for 10 minutes to pellet the nuclei. The supernatant was spun down at 230,000 g_{av} for 60 minutes in the Spinco L2 65B ultracentrifuge to obtain the *cytosol fraction*. The crude nuclear fraction was washed with medium A and suspended 35 ml of medium B (2.4 M surcose, 3 mM $CaCl_2$). This suspension was layered over 5 ml of medium B and centrifuged in the Spinco SW 27 rotor at 22,500 rpm for 60 min (Liao et al., 1973). Purified nuclei were washed twice with 30 ml of medium C (5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM mercaptoethanol, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5) and suspended in medium C containing 1 M NaCl (Rennie, Bruchovsky, 1972). This suspension was

allowed to stand overnight at +4°C. Finally, the nuclei were disrupted by sonication (ARTEK, Sonic 300 Dismembrator, medium tip, intensity setting: 60, continuous shaking for 2 min at 0°C) to yield the homogenized *nuclear fraction.*

Processing of the cytosol fraction – The cytosol fraction was subjected to equilibrium dialysis against 30 volumes of medium D (2 mM mercaptoethanol, 20 mM Tris-HCl, pH 7.5) at +5°C for 45 hours.

Protein concentration in dialysed cytosol was determined (Lowry et al. 1951) and steroids from an aliquot of the dialysed cytosol ("inside bag fraction") and the dialyzing buffer ("outside bag fraction") were quantitatively extracted with 2×3 vol. diethylether and 1×3 vol. chloroform. We found that unlabelled dihydrotestosterone added to the dialyzing buffer during dialysis did not displace ^{3}H-dihydrotestosterone bound to cytosol proteins.

Processing of the nuclear fraction – Aliquots were taken for the determination of DNA (Dische, 1955) and protein contents. In protein determinations horse serum albumin, in DNA measurements salmon sperm DNA served as standards. The rest of the nuclear fraction was successively shaken with 1×3 vol. diethylether, 2×3 vol. chloroform and 3×3 vol. dichloromethane. The combined extracts contained more than 99 per cent of the label extractable by organic solvents.

Thin-layer chromatography – Steroid extracts obtained from dialysed cytosol, dialysing buffer and nuclear fraction were evaporated to dryness under vacuum and the residue was subjected to chromatography on a Kieselgel HF_{254} (Merck, Darmstadt, GFR) thin-layer plate using chloroform–diethylether 90 : 10 (v/v) or 85 : 15 (v/v) mixture as solvent system (Anderson, Liao 1968). Unlabelled testosterone, 5α-dihydrotestosterone and occasionally 5α-androstane 3β,17β-diol and 5α-androstanedione were applied as carriers. Steroid spots were localized in ultraviolet light and in iodine vapour. Gel segments carrying spots were scraped off into counting vials, shaken with 1 ml methanol for 2 min and after addition of 10 ml of a scintillation cocktail, the radioactivity was measured.

It should be noted that the thin-layer chromatography applied in this study works very well for the separation of testosterone, dihydrotestosterone, androstanediols and androstanedion. It does not separate, however, 5α-dihydrotestosterone from 5β-dihydrotestosterone and androsterone. Available evidence indicates that metabolization of testosterone into these steroids is only of marginal significance in the seminal vesicles (Buric et al., 1972). The scheme of the overall procedure is shown in Fig. 1.

Measurement of radioactivity – Scintillation cocktail contained 0.4% PPO, 0.005% POPOP in toluene. Radioactivity was measured with 43% efficiency using a Packard Tri-Carb 2425 spectrometer.

Calculations – Radioactivity bound to cytosol proteins and to nuclei was calculated in cpm/mg protein and cpm/mg DNA, respectively. In some cases the means ± S.D. of data were also calculated.

Steroids – 1,2,6,7 (n)^{3}H-testosterone (87 Ci/mmol) was the product of The Radiochemical Centre, Amersham, England. 19-Nortestosterone was kindly sup-

plied by Richter Chemical and Pharmaceutical Works, Budapest. All the other steroids were obtained from Merck, Darmstadt, GFR.

Results

Testosterone metabolism and dihydrotestosterone binding – The standard reaction mixture containing 5×10^{-9} M ^{3}H-testosterone and the mince of the seminal vesicles was kept ice cold, equilibrated in oxygen stream for 20 min and incubated at 37°C. Incubations were carried out for various periods, up to 60 min, and the mince of the seminal vesicles was processed as described under Materials and methods. Figure 2 shows that the binding of dihydrotestosterone to cytosol proteins was most rapid during the first 10 min of incubation. On the other hand, accumulation of dihydrotestosterone by nuclei followed a different time course. The level of this metabolite of testosterone increased progressively during the first 40 min and reached a final value during the last 20 min of incubation.

In addition to dihydrotestosterone, considerable amounts of testosterone also accumulated in the nuclei. The time course of testosterone binding, however, was very different from that of dihydrotestosterone. It showed an initial sharp rise with a maximum at about ten minutes, which was followed by a progressive decline.

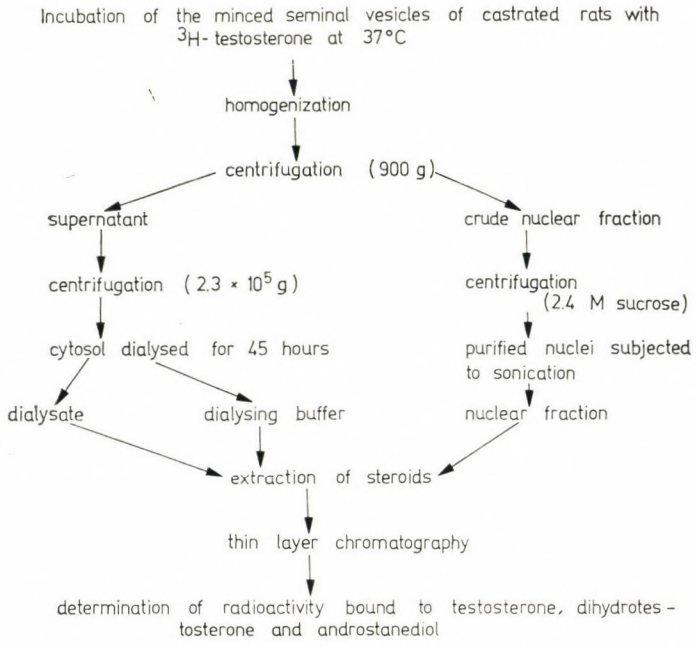

Fig. 1. Scheme of the experimental procedure

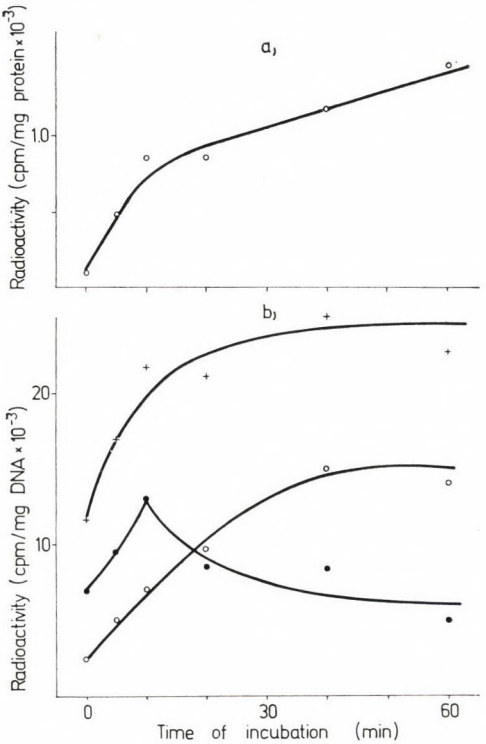

Fig. 2. Time course of the binding of testosterone and dihydrotestosterone to cytosol proteins and nuclei of the seminal vesicle. Mince of the seminal vesicles from rats castrated 6 days before the experiment was added to Krebs-phosphate medium containing 5 nM ^{3}H-testosterone. The mixture was kept ice cold while equilibrated in oxygen stream for 20 min. Five mixtures of this type were incubated at 37 °C for various periods as indicated in the diagram. Vesicular tissues were fractionated and fractions were processed by the standard procedures. –○–○– Radioactivity in dihydrotestosterone, –●–●– radioactivity in testosterone, –×–×– total radioactivity a) cytosol proteins; b) nuclei

Figure 3 shows the change of the levels of testosterone and its metabolites in the "outside bag" cytosol fraction (which contains the steroids not bound to proteins). It can be seen that at 37 °C testosterone is progressively metabolized into dihydrotestosterone and androstanediols.

Dependence of dihydrotestosterone binding on the concentration of ^{3}H-testosterone – Figure 4 demonstrates that as low as 6×10^{-9} M ^{3}H-testosterone in the medium was sufficient to saturate cytosolic and nuclear binding sites with dihydrotestosterone when the incubation was carried out for 60 min. It can also be seen that half saturation of these binding sites was achieved at about 10^{-9} M testosterone concentration. Most of the label recovered from the nuclei was identifiable in dihydrotestosterone but small amounts of ^{3}H-testosterone were found at all testosterone concentrations studied.

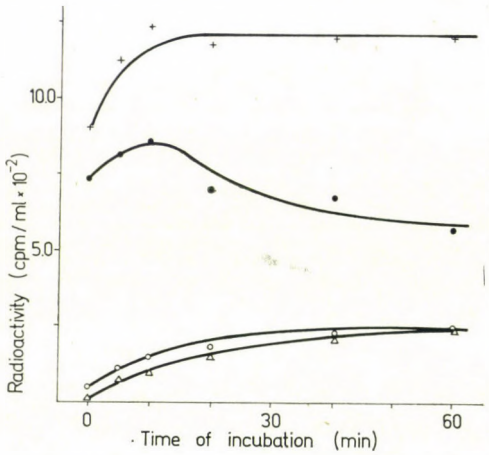

Fig. 3. Metabolization of testosterone in the seminal vesicles. The "outside bag" cytosol fractions from the experiment described in Fig. 2 were analyzed for labeled steroids by thin-layer chromatography. —○—○— Radioactivity in dihydrotestosterone, —●—●— radioactivity in testosterone, —△—△— radioactivity in androstanediols, —×—×— total radioactivity

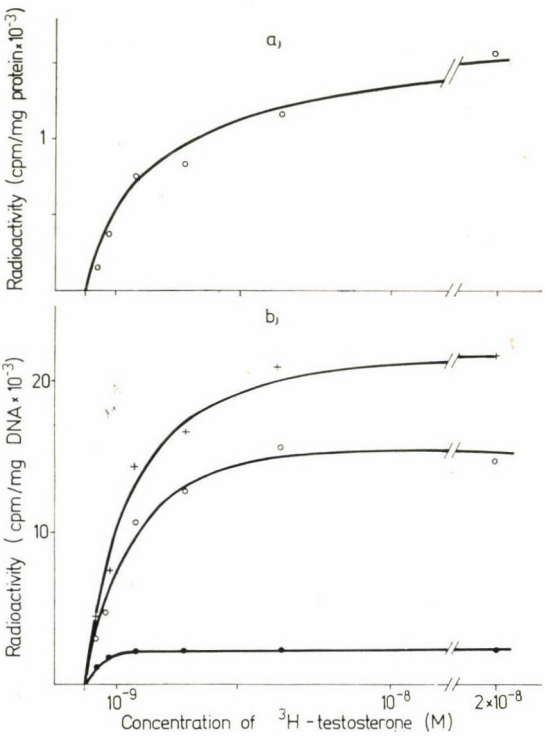

Fig. 4. Binding of testosterone and dihydrotestosterone as a function of the concentration of testosterone in the incubation medium. Incubations were carried out for 60 min. Otherwise, experimental conditions were the same as in Fig. 2. For symbols see legend to Fig. 2

Figure 5 shows the distribution of radioactivity in the "outside bag" cytosol fractions obtained in the experiment demonstrated in Fig. 4. With increasing testosterone concentrations the metabolites of this steroid were also found in increasing amounts, indicating that the enzymes which convert testosterone to dihydrotestosterone and androstanediols were not saturated with their respective steroid substrates. It is improbable, therefore, that the binding of dihydrotestosterone to cytosolic and nuclear sites reflects a binding of this steroid to some of the enzymes acting on steroids. The dependence of nuclear binding of testosterone and dihydrotestosterone on the concentration of testosterone in the incubation medium was also studied using 10 min incubation times. As shown in Fig. 6, in contrast to the accumulation of dihydrotestosterone, the uptake of testosterone is not a saturable process, at least when the testosterone concentrations fall in the range of 10^{-9}–10^{-7} M. During 10 min incubation at 37°C about 4×10^{-8} M testosterone was necessary to saturate cytosol receptors and nuclei with dihydrotestosterone.

Quantitative aspects of dihydrotestosterone binding – Table 1 shows that after incubation of the vesicular mince at a saturating (10 nM) testosterone level for 1 hour, the label attached to cytosol proteins and to nuclei was predominantly in dihydrotestosterone. This binding is selective, since among the free steroids of the cytosol the proportion of dihydrotestosterone was only about 30%. Under the above conditions nuclei were found to bind 1 pmol dihydrotestosterone per g tissue. Taking into account that one nucleus in rat tissues contains 9.6 pg DNA (Venderly, 1955), this amount may correspond to 1900 binding sites per nucleus. The amount of dihydrotestosterone bound to cytosol proteins was relatively small, 0.4 pmole/g tissue (Table 1).

Table 1

Fractions of nuclear and cytosolic radioactivity recovered in dihydrotestosterone following incubation of mince of the seminal vesicles with ^{3}H-testosterone

Mince of the seminal vesicles obtained from rats castrated 6 days before killing was incubated at 37°C with 10 nM ^{3}H-testosterone for 1 hour

	Total dpm/g tissue	Dihydrotestosterone	
		% of total dpm	pmol/g tissue
Nuclei*	$2.33 \times 10^5 \pm 3.1 \times 10^4$	83 ± 4	1.0
Cytosol			
-free	$1.35 \times 10^6 \pm 1.65 \times 10^5$	29 ± 3	2.0
-bound	$1.05 \times 10^5 \pm 1.2 \times 10^4$	73 ± 11	0.4

* Corrected for DNA loss during preparation of nuclear fraction
Mean ± S.D.; n = 6

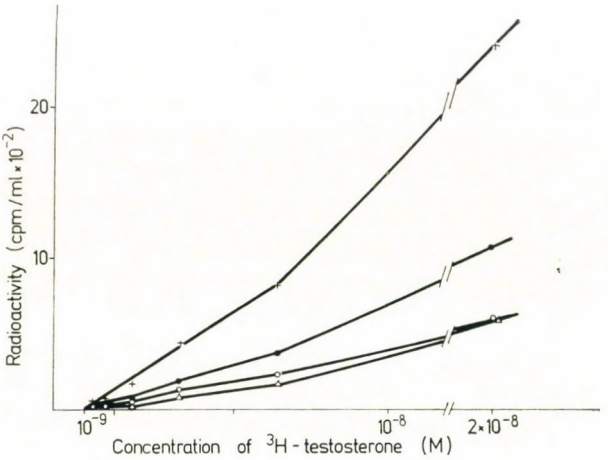

Fig. 5. Metabolization of testosterone at various concentrations of testosterone in the incubation medium. "Outside bag" cytosol fractions obtained in the experiment described in Fig. 4 were analyzed for labeled steroids by thin-layer chromatography. For symbols see the legend to Fig. 3

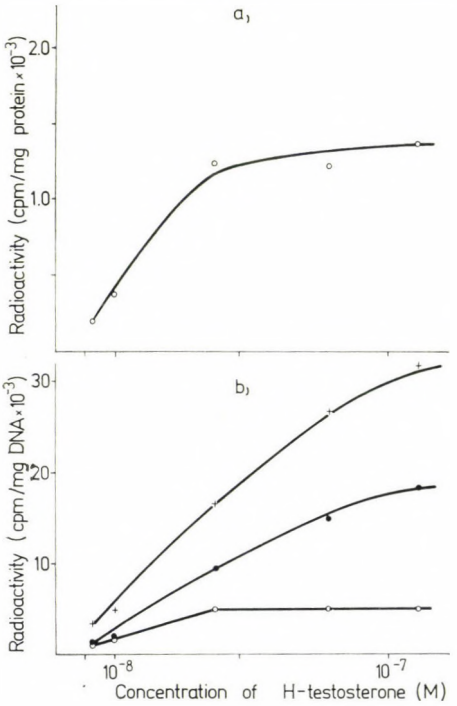

Fig. 6. Binding of testosterone and dihydrotestosterone to cytosol proteins and to nuclei after 10 min incubation of vesicular mince with various concentrations of testosterone. Incubations were carried out for 10 min, other conditions were identical with those described in Fig. 2. For symbols see the legend to Fig. 2

Estimation of "aspecific" binding sites – In order to test the extent of binding of dihydrotestosterone to "aspecific" binding sites (i.e. to sites which bind dihydrotestosterone with low affinity and high capacity) we carried out isotope dilution experiments. In a control vessel the mince of the seminal vesicles was incubated with 10 nM ^{3}H-testosterone while in the test vessels the labeled steroid was diluted

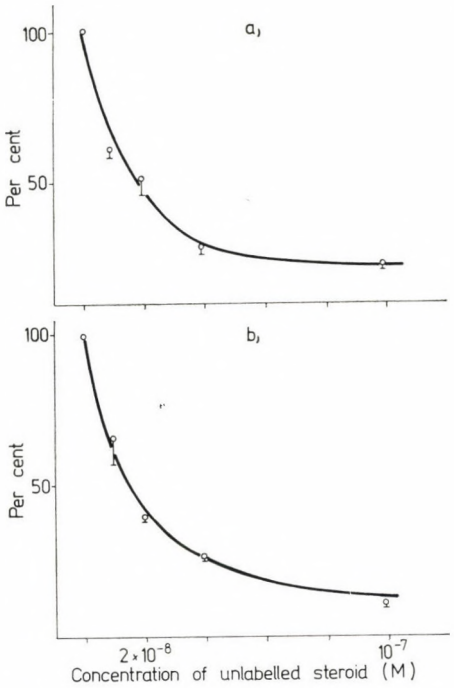

Fig. 7. Effect of dilution of ^{3}H-testosterone with various concentrations of unlabeled testosterone on the binding of ^{3}H dihydrotestosterone to cytosol proteins and to nuclei. Various concentrations of unlabeled testosterone were mixed with 10 nM ^{3}H-testosterone in the incubation medium before the addition of vesicular mince. The flasks were kept ice cold and perfused with oxygen for 20 min. Incubations were carried out for 60 min at 37 °C. Data were calculated as percentage of the controls which did not contain unlabeled testosterone. The mean values ± S.D. obtained from 4 separate experiments are presented a) cytosol proteins; b) nuclei

with various concentrations of unlabeled testosterone. After 1 hour incubation at 37 °C the binding of ^{3}H-dihydrotestosterone to cytosol proteins and to nuclei was determined by the standard procedure. Counts measured in the presence of unlabelled steroid (Fig. 7) were compared with the values expected theoretically. It was calculated that in the case of the cytosol proteins "aspecific" binding amounted to about 15% of the control value, while in the case of the nuclei it was about 5%.

The relationship between nuclear testosterone and dihydrotestosterone – The time course of the uptake of testosterone and of the accumulation of dihydrotestosterone in the nuclei (Fig. 2) could be interpreted by assuming that nuclear dihydrotestosterone emerged simply from nuclear metabolization of testosterone to its 5α-dihydro derivative. The following experiments suggest, however, that these processes are more complex than that.

(I) In contrast to the accumulation of dihydrotestosterone, the uptake of testosterone cannot be saturated in the range of $10^{-9}-10^{-7}$ M testosterone concentration (Fig. 6).

(II) When animals were given testosterone in the form of intraperitoneal injection 1 or 2 hours prior to killing, the binding of dihydrotestosterone was suppressed while that of the testosterone did not change substantially (Table 2).

Table 2

Effects of testosterone injection on the in vitro binding of dihydrotestosterone and testosterone to nuclei and cytosol proteins

Each group consisted of 6 animals castrated 6 days prior to the experiment. The vesicular mince from each group was incubated with 10 nM ^{3}H-testosterone for 60 min. Determination of steroid binding was as described in the experimental section. Testosterone phenylpropionate (TPP) was dissolved in 0.2 ml of an ethyleneglycol–ethanol 1 : 1 (v/v) mixture and administered intraperitoneally. Control animals received the vehicle only

	Cytosol, cpm/mg protein	Nuclei		
		total	dihydro-testosterone, cpm/mg DNA	testosterone
Control	1207	18 626	15 250	1089
	1102	17 704	13 320	1254
1 mg TPP, 1 hour before killing	816	13 863	10 200	1123
1 mg TPP, 2 hours before killing	689	12 562	9 050	1432

(III) When added to the incubation mixture, 19-nortestosterone (which is known to be less androgenic than testosterone) and unlabeled testosterone were equally effective in suppressing testosterone uptake of nuclei, while 19-nortestosterone was only half as potent as testosterone in suppressing ^{3}H-dihydrotestosterone binding to cytosol proteins and to nuclei (Table 3). This latter difference in competitive action could not be ascribed to a difference in the rate of conversion of testosterone to dihydrotestosterone (Table 3). All these results suggest that vesicular nuclei bind testosterone and dihydrotestosterone by independent mechanisms.

Table 3

Binding of ^{3}H-testosterone and ^{3}H-dihydrotestosterone to cytosol proteins and nuclei in the presence of 19-nortestosterone or unlabelled testosterone

Vesicular mince was incubated at 37 °C with 10 nM ^{3}H-testosterone for 60 min in the presence of 40 nM competitor steroid. Control mixtures were incubated without competitors. Steroids bound to cytosol proteins and to nuclei were determined as described in Materials and methods

	Competitor steroid added	
	testosterone	19-nor-testosterone
^{3}H-dihydrotestosterone bound to cytosol proteins*	29 ± 2	55 ± 4
^{3}H-dihydrotestosterone bound to nuclei*	27 ± 2	49 ± 6
^{3}H-testosterone bound to nuclei*	36 ± 7	36 ± 8
% free ^{3}H-dihydrotestosterone**	24 ± 1	25 ± 1

mean ± S.D.; n = 4
* given as per cent of control (control value = 100%)
** measured in "outside bag" cytosol fraction

Discussion

In previous studies from our laboratory the effect of testosterone on the protein synthesis of rat seminal vesicle was investigated using glands obtained from animals killed 5 or more days after orchidectomy (Mányai, 1964b; Tóth, Mányai, 1968b; Tóth, Machovich, 1969; Tóth, 1970;1974). Accordingly, we selected for our receptor studies the vesicles of animals castrated 5 or 6 days before the experiment was started.

Under our *in vitro* experimental conditions testosterone was metabolized very similarly to that found in *in vivo* experiments (Buric et al., 1972). The main products were dihydrotestosterone and the androstanediols. This metabolic pattern is analogous to that reported for the prostate (Fang et al., 1969).

Experiments with rat prostate and seminal vesicle have revealed that dihydrotestosterone can be formed from testosterone in the microsomal membranes and in the nuclei, whereas the site of formation of the androstanediols is the cytoplasm (Bruchovsky, Wilson, 1968; Morfin et al., 1970; Wilson, Gloynd, 1970). Following castration of adult rats most of the endoplasmic reticulum disappears from the vesicular cells during one week or so (Moore et al., 1930, Tóth, 1974). Therefore a testosterone reductase activity of the nuclei might particularly be important to promote the action of testosterone in the seminal vesicles of rats castrated 5–6 days before the administration of the hormone.

Our finding that cytosol proteins and nuclei can selectively bind a limited

number of dihydrotestosterone molecules with high affinity demonstrates the presence of androgen receptors in these compartments of the vesicular cell. It should be noted that the methods employed in this study did not make it possible to discriminate between androphil proteins having similar binding affinity but different functional significance. In the prostate, at least two types of cytosol proteins (termed α- and β-protein) could be distinguished which bound dihydrotestosterone with high affinity (Fang, Liao, 1971, Katsumata, Goldman, 1974). Only β-protein was capable of transferring dihydrotestosterone to nuclei, while the complex of α-protein with dihydrotestosterone proved even to be inhibitory for this process.

Both the cytosol and the nuclear binding sites were saturated with dihydrotestosterone in our experiments when the medium contained as little as 6 nM testosterone. Considering that dihydrotestosterone amounts to about 25 per cent of the free steroids found in the cytosol fraction, it can be calculated that about 1.5 nM dihydrotestosterone is necessary to saturate these binding sites. This value closely resembles binding data obtained with cytosol prepared from the prostate (Fang et al., 1969). On the other hand, when a mince of the prostate obtained from normal rats or from rats castrated 6 days earlier was used for binding studies, the nuclei could only be saturated at about 30-fold higher testosterone concentration (Fang et al., 1969). This difference in the binding characteristics of the vesicular and prostatic mince deserves attention, since the seminal vesicles have been observed to respond *in vivo* to testosterone more sensitively than the ventral prostate (Price, Williams-Ashman, 1961). This difference cannot be explained in terms of a difference in steroid metabolism or a different affinity of dihydrotestosterone to cytosol receptors. It is conceivable, however, that the seminal vesicles are devoid of proteins which can counteract the transfer of dihydrotestosterone to nuclei. As we mentioned before, the presence of such a protein in the prostate has been demonstrated.

According to our calculations a nucleus in the seminal vesicles contains about 1900 binding sites for dihydrotestosterone. About the same figure has been reported by Fang et al. (1969) for prostatic nuclei. As many as 3400 and 6000 binding sites per nucleus of a prostate cell have been found by Rennie and Bruchovsky (1972) and Mainwaring and Peterken (1971), respectively. It appears that, compared to the values reported for prostatic nuclei, nuclei of the seminal vesicles can bind an identical or $2-3$ times less amount of dihydrotestosterone. On the other hand, the maximal amount of dihydrotestosterone (0.4 pmol/g tissue) bound to the vesicular cytosol proteins is much less than the binding (20 pmol/g tissue) reported for the cytosol prepared from the prostate 1 day after castration (Rennie, Bruchovsky, 1973). Our finding fits in with that of Bruchovsky and Craven (1975) that the receptor activity of prostatic cytosol (but not that of the nuclei) decreased during the first week after castration to very low levels. It should be noted that levels of androgen–receptor complexes as low as 0.7 pmol/g tissue are sufficient to saturate nuclear binding sites in the prostate (Rennie, Bruchovsky, 1973).

The time course of the binding of dihydrotestosterone to cytosol proteins and cell nuclei of the seminal vesicles resembles closely those described for various prostatic systems (Mainwaring, Peterken, 1971; Rennie, Bruchovsky, 1972; 1973; Nozu, Tamaoki, 1974). The cytosol proteins are predominantly labelled in the first 10 min of incubation, while the accumulation of dihydrotestosterone in the nuclei is a slower process and attains a maximum after 40 min incubation. These time relations are compatible with the existence of a "two step" binding mechanism (Jensen et al., 1968) in the vesicles: the dihydrotestosterone is attached first to the cytosol receptor and subsequently transferred as a receptor–steroid complex to the nucleus.

It is striking that in the vesicular tissue incubated *in vitro* with testosterone, the nuclei take up considerable amounts of testosterone. It is an obvious possibility that nuclei accumulate testosterone which is transformed in them to dihydrotestosterone. Such a mechanism has been suggested for the prostate (Rennie, Bruchovsky, 1973). Our results favor the concept that the binding of testosterone and dihydrotestosterone in the nuclei takes place by independent mechanisms. Our results indicate that nuclear binding of testosterone is characterized by a much higher half-saturation value than the binding of dihydrotestosterone. Moreover, it appears that 19-nortestosterone and testosterone are bound to nuclei with similar affinity but the binding of dihydrotestosterone to nuclei is significantly tighter than that of 19-nortestosterone. Cytosol proteins also have a higher affinity to dihydrotestosterone than to 19-nortestosterone. A nuclear testosterone 5α-reductase present in the prostate of the rat has been reported to have a comparatively high apparent K_m value (3×10^{-5}M) which is identical for testosterone and 19-nortestosterone (Shimazaki et al., 1971). In our experiments testosterone in the vesicles might have been bound to a similar nuclear enzyme. One may speculate that dihydrotestosterone molecules produced in the nuclei or in the outer nuclear membrane can migrate into the cytoplasm where they become attached to cytosol receptors and are transferred then to specific nuclear binding sites in the form of a receptor–steroid complex. It should be noted that 19-nortestosterone (and/or its derivatives) is bound to cytosol proteins and nuclei less firmly than dihydrotestosterone, which is in accordance with the lower androgenicity of this steroid.

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References

Anderson, K. M. Liao, S. (1968) Nature *219* 277
Bruchovsky, N., Craven, S. (1975) Biochem. Biophys. Res. Commun. *62* 837
Bruchovsky, N., Wilson, J. D. (1968) J. Biol. Chem. *243* 2012
Buric, L., Becker, H., Petersen, C., Voigt, K. D. (1972) Acta Endocrinol. (Kbh.) *69* 153
Dische, Z. (1955) In: The Nucleic Acids, Vol. 1. Chargaff, J. N. Davidson (Eds). Acad. Press, New York—London, p. 285

Fang, S., Anderson, K. M., Liao, S. (1969) J. Biol. Chem. *244* 6584
Fang, S., Liao, S. (1971) J. Biol. Chem. *246* 16
Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., De Sombre, E. R. (1968) Proc. Nat. Acad. Sci. U. S. *59* 632
Katsumata, M., Goldman, A. S. (1974) Biochim. Biophys. Acta *359* 112
King, R. J. B., Mainwaring, W. I. P. (1974) In: Steroid–Cell Interactions. Butterworths, London, p. 41.
Krebs, H. A. (1950) Biochim. Biophys. Acta *4* 249
Liao, S., Liang, T., Fang, S., Castaneda, E., Shao, T. (1973) J. Biol. Chem. *248* 6154
Lowry, O. H., Rosenbrough, N I., Farr, A. L., Randall, A. L. (1951) J. Biol. Chem. *193* 265
Mainwaring, W. I. P., Irving, R. (1973) Biochem. J. *134* 113
Mainwaring, W. I. P., Peterken, B. M. (1971) Biochem. J. *125* 285
Mányai, S. (1964a) Acta Physiol. Acad. Sci. Hung. *24* 419
Mányai, S. (1964b) Acta Physiol. Acad. Sci. Hung. *24* 11
Mányai, S., Beney, L., Czuppon, A. (1965) Acta Physiol. Acad. Sci. Hung. *28* 105
Moore, C. R., Gallagher, T. F., Hughes, W. (1930) Am. J. Anat. *45* 109
Morfin, R. F., Aliapoulios, M. A., Chamberlain, J., Ofner, P. (1970) Endocrinology *87* 394
Nozu, K., Tamaoki, B. (1974) Biochem. Biophys. Res. Commun. *58* 145
Price, D., Williams-Ashman, H. G. (1961) In: Sex and Internal Secretions. Vol. 1. W. C. Yount (Eds). The Williams and Wilkins Co., Baltimore, p. 366
Rennie, P., Bruchovsky, N. (1972) J. Biol. Chem. *247* 1546
Rennie, P., Bruchovsky, N. (1973) J. Biol. Chem. *248* 3288
Sar, M., Liao, S., Stumpf, W. E. (1970) Endocrinology *86* 1008
Shimazaki, J. Horaguchi, T., Ohki, Y., Shida, K. (1971) Endocrinol. Japon. *18* 179
Stern, J. M., Eisenfeld, A. J. (1969) Science *166* 233
Tóth, M. (1970) FEBS Letters *8* 337
Tóth, M. (1974) Acta Biol. Med. Germ. *33* 845
Tóth, M., Machovich, R. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 339
Tóth, M., Mányai, S (1968a) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 29
Tóth, M., Mányai, S. (1968b) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 337
Tóth, M., Zakár, T. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. *6* 231
Tveter, K. J., Unhjem, O. (1969) Endocrinology *84* 963
Venderly, R. (1955) In: The Nucleic Acids, Vol. 2. E. Chargaff J. N. Davidson (Eds). Acad. Press, New York—London, p. 155
Wilson, I. D., Gloynd, R. E. (1970) In: Recent Progress in Hormone Research, Vol. 26. E. B. Astwood (Ed.) Acad. Press, New York—London p. 309

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Fate of Foreign DNA Taken up by Tonsillar Lymphocytes

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The uptake of heterologous (*B. cereus*) DNA by tonsillar lymphocytes was followed for 22 hours. Acid-precipitable DNA became associated with the cells as soon as they were mixed with DNA. The uptake was linear from 0 to 3 hours, between 3 to 6 hours part of the cell-associated DNA was released into the incubation medium, and at 22 hours the amount of label in the cells reached the values measured at 2—3 hours. In respect of the size of DNA the uptake seemed to follow the same saturation curve. There was no difference between the uptake pattern of native DNA and that of heat-denatured DNA. The decrease of size of foreign DNA in the cells and in the incubation medium was followed by agarose-gel electrophoresis. In tonsillar lymphocytes enzyme activities capable of degrading heterologous DNA were found. The enzyme activities present in the cells could be detected in the culture medium as well for at least 24 hours.

Introduction

The mechanism and kinetics of DNA uptake, as well as the fate and degradation of foreign DNA by mammalian cells are poorly understood (Bhargava, Shanmugam, 1971). It is generally accepted that "uptake" of a nucleic acid by cells means the removal of these macromolecules from the medium and includes both the binding of the nucleic acid to the cell surface and its transport into the cell. DNA uptake and transformation in bacteria have been extensively studied (Natani, Setlow, 1974). Although numerous studies dealt with DNA uptake and the fate of exogenous DNA in eukaryotic cells, there are still several points which await further elucidation. Foreign DNA has to pass through several membranes and find its way through a number of cell components before it reaches the nucleus; on its way the DNA may be exposed to degradation by naturally occurring endo- and exonucleases (Ledoux, 1965).

In order to study DNA uptake, it was necessary to consider the properties of the receptor cells and the size of the DNA molecules. To simplify cell complexity, primary tonsillar lymphocytes were used. These cells, after 20 hours of culturing, are known to be converted into small resting lymphocytes (Staub, 1974). We hoped that by the use of these cells we could avoid at least the cell cycle and the problem of cell aging. A number of authors (Bhargava, Shanmugam, 1971)

have demonstrated that foreign DNA taken up by eukaryotic cells is degraded to acid-soluble material only to a small extent. However, these observations did not extend beyond 2 or 3 hours. DNA degradation during prolonged incubation has not yet been explored in detail.

Materials and methods

Preparation of (^3H) DNA from B. cereus

DNA was prepared according to the method of Saito and Miura (1963) with some modifications. When the O.D. at 620 nm of the *B. cereus* 130 thy$^-$ (^{3}H thymin 2 μCi/ml) culture reached a value of about 1.5 the cells were centrifuged, washed once with distilled water at 60 °C (in order to stop sporulation and deoxyribonuclease activity), and once with 0.5% KCl – 0.5% NaCl solution. The pellet was treated with lytic enzyme prepared from *B. cereus* (Csuzi, 1971) for 30 min at 37 °C, then the Saito and Miura procedure was followed. The DNA obtained had a molecular weight of about 10^7 daltons, its specific activity was 4 – 5000 cpm/nmol and the concentration of acid-soluble material was below 0.5%. For denaturation, the labelled DNA was heated in a boiling water bath for 10 min and quenched on ice.

Cell culture and media

Human tonsillar lymphocytes were obtained (Piffko et al., 1970) from tonsils surgically removed from 2 – 6 years old children. The lymphocytes were suspended in Eagle's medium (MEM) (Eagle, 1959), supplemented with 10% bovine serum, 100 units penicillin and 100 μg streptomycin/ml, and incubated at 37 °C at a concentration of 2×10^6 cell/ml.

Inoculation of lymphocytes with (^3H) labelled B. cereus DNA

After 13 hours of cultivation when almost all of the cells were small lymphocytes (Staub, 1974), the cells were centrifuged at $1000 \times g$ for 5 min and washed twice with Hanks' solution in order to remove the serum, cellular debris and cellular components released into the medium. The pellet was suspended in MEM containing antibiotics at a concentration of 10^7 cells/ml, and distributed in 1 ml portions into culture tubes. Labelled *B. cereus* DNA was added at different concentrations. The adsorption of DNA to the cells was allowed to proceed for different time periods between 0 and 22 hours at 37 °C. The inoculum was not removed. At the end of the adsorption period, the incubation was stopped by the addition

of 1 ml incubation medium containing 0.05 M sodium iodoacetate, pH 7.3, in the cold (Meizel, Kay, 1964). The cells were spun down at 1 000×*g* for 5 min and washed twice with incubation medium containing 0.025 M sodium iodoacetate, pH 7.3.

Measurement of (^3H) DNA uptake

A 1 ml aliquot of the supernatant was treated in the cold with 1 ml 1 M perchloric acid (PCA). The cell pellet, after lysis with 1 % sodium dodecyl sulfate (SDS) in 0.01 M Tris buffer, pH 7.5, was precipitated with 0.5 M PCA in the cold. After centrifugation, radioactivity of the supernatant's acid-soluble fraction was measured. The radioactivity of the acid-insoluble material was measured in the pellets after hydrolysis with 0.5 M PCA at 90 °C for 30 min.

Preparation of lymphocyte cell extracts and enzyme assay

Cell extracts were prepared as follows: 10^7 lymphocytes suspended in 1 ml 0.01 M Tris, buffer pH 7.5, 0.01 M KCl, 0.002 M $MgCl_2$ were disrupted in a glass Dounce homogenizer. After centrifugation at 10 000 ×*g* for 30 min the supernatant was used for enzyme assay.

Nuclease assay was performed at 37 °C. The reaction mixture contained 0.05 ml cell extract, 0.05 ml (^3H) *B. cereus* DNA (100 μg) and 0.1 ml proper buffer: (1) pH 4.5 = 0.1 M Na acetate containing 1 mM $MgCl_2$, (2) pH 7.4 = 0.05 M Tris-HCl containing 1 mM $MgCl_2$ and 10 mM $CaCl_2$, (3) pH 8.5 = 0.05 M Tris-HCl containing 1 mM $MgCl_2$.

Agarose-gel electrophoresis

Changes in the size of (^3H) DNA were detected by agarose-gel electrophoresis as described by Helling et al. (1974). Samples taken from different reaction mixtures were treated with pronase and SDS at a final concentration of 500 μg/ml and 1 %, respectively, at 30 °C for 30 min. DNA was extracted two times with 0.05 M Tris-HCl (pH 9) saturated phenol. The phenol was removed with diethyl ether. Each sample (0.1 ml) was mixed with 0.05 ml of 20 % sucrose and put on the agarose-gel (10 cm × 0.6 cm) fixed with dialyzing membrane on the bottom to retain the gel and the smaller DNA fragments. The samples were run for 5 min at 100 V and thereafter at 1.5 V/cm gel for 18 hours.

Determination of radioactivity

(^3H) activity in the samples was measured in 10 ml of toluene–ethanol (1 : 1) scintillation cocktail (PPO 4 g/l and POPOP 0.05 g/l) in a Beckman Scintillation Spectrometer.

(^{3}H) thymine (14 Ci/nmol) was purchased from the Isotope Institute, Hungarian Academy of Sciences.

Results

The kinetics of DNA uptake in a representative experiment are shown in Table 1. Tonsillar lymphocytes were incubated with native *B. cereus* (^{3}H) DNA at a concentration of 150 μg DNA (10^7 cells in 1 ml of MEM.) Acid-precipitable radioactivity became cell associated as soon as the cells were mixed with the foreign DNA, in spite of the use of sodium iodoacetate to remove non-specifically bound DNA (Meizel, Kay, 1964). During the first 2 – 3 hours these values increased, followed by a decrease. The amount of (^{3}H) DNA in the cells after 22 hours of incubation again reached the value observed at 2 hours. In Table 1 we also show the radioactivity of the acid-insoluble fraction in the supernatant at different times during incubation. A good correlation was found between the increase in the amount of DNA in the cells and the decrease of DNA in the incubation medium. The amounts of acid-soluble labelled DNA present in the same samples are given in Table 2. The highly polymerized DNA initially contained only 0.25% acid-soluble material that increased slightly with a constant rate, reaching 1 – 1.5% of the total radioactivity after 22 hours of incubation.

The pattern of uptake from increasing doses of labelled foreign DNA was measured after 2 and 22 hours of incubation. As shown in Fig. 1, a direct correlation was found between the uptake and the given dose. This correlation followed a saturation curve.

In the following experiments we compared the uptake of native and heat-denatured *B. cereus* (^{3}H) DNA by tonsillar lymphocytes. The results are given in Table 3. The course of DNA uptake was followed for 22 hours. The amount of cell-associated acid-insoluble radioactivity was nearly the same irrespective of whether native or heat-denatured DNA was added to the lymphocyte suspension.

Table 1

Kinetics of uptake of B. cereus DNA by lymphocytes

Time, hours	Lymphocyte cells	Supernatant
	cpm/ml	cpm/ml
0	14 102	31 136
2	17 085	26 414
3	15 312	30 958
5	9 734	36 084
6	9 493	27 164
22	18 378	24 443

Table 2

Acid-soluble labelled DNA in lymphocytes and in the supernatant after incubation with (^3H) B. cereus DNA

Time, hours	Lymphocyte cells		Supernatant	
	cpm/ml	%	cpm/ml	%
0	88	0.25	208	0.26
2	155	0.44	225	0.30
3	158	0.45	288	0.38
5	175	0.50	395	0.52
22	503	1.43	1035	1.38

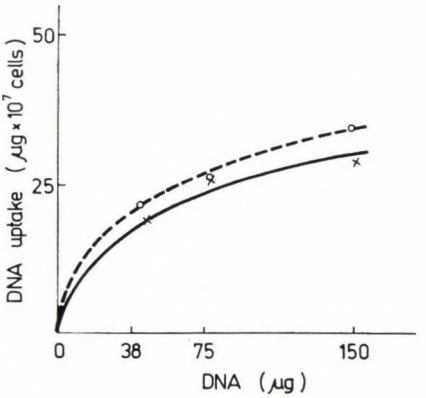

Fig. 1. DNA uptake by lymphocytes incubated with increasing doses of labelled *B. cereus* DNA. DNA uptake assayed as the amount of acid insoluble fraction after 2 hours (× — ×) and after 22 hours (× - - - - ×) of incubation

Table 3

Kinetics of uptake of native and denatured B. cereus DNA by lymphocytes

Time, hours	10^7 Lymphocyte cells/ml	
	Native DNA, cpm/ml	Denatured DNA, cpm/ml
0	4473	2984
1	5051	—
2	3382	5204
4	3546	4764
5	1843	3532
23	3607	5794

In eukaryotic cells various deoxyribonucleases have been described (Laskowski, 1967, Zölner et al., 1974). In order to study the fate of DNA, the size of labelled DNA taken up by lymphocytes was estimated by agarose-gel electrophoresis. Fig. 2a illustrates the distribution of labelled DNA extracted from the cells at 0, 2, 5 and 22 hours of incubation.

Figure 2b indicates the distribution of radioactivity in the samples taken from the supernatants at the same times. The data show that the degradation of DNA went parallel in the cells and in the medium.

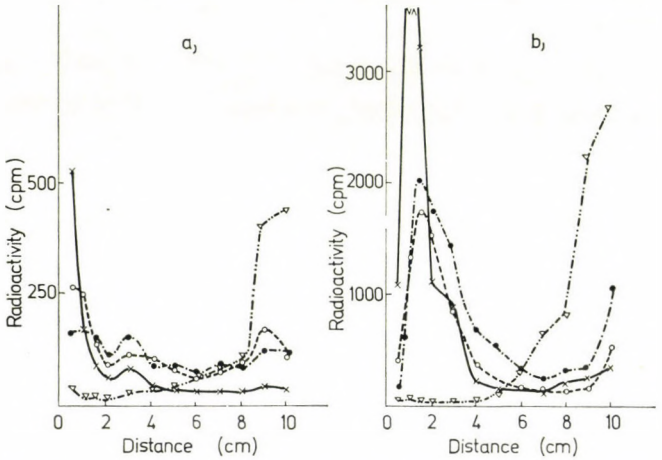

Fig. 2. Agarose-gel electrophoresis of DNA after different times of incubation with lymphocytes; 100 μg/ml native *B. cereus* DNA was incubated and treated as described in Materials and methods. a) Pattern of (^{3}H) DNA obtained from the cells. Incubation time: × — ×, 0 h; ○ - - - ○, 2 hours; ● - . - . - ●, 5 hours; △ - . - . △, 22 hrs. b) Pattern of (^{3}H) DNA obtained from the supernatant. Incubation time: × — ×, 0 h; ○ - - - ○, 2 hrs; ● . - . - . ●, 5 hrs; △ . . - . . △, 22 hrs

The presence of deoxyribonucleases was also studied in cell extracts incubated with labelled DNA at different pH values. The data presented in Fig. 3b indicate that only a small change in the molecular size could be detected even after 5 hours of incubation at pH 7.4. However, considerable DNA degradation was found under acidic and alkaline conditions (Figs 3a and 3c).

The occurrence of fragmented DNA in the incubation medium may be due to a release of degradative enzymes into the medium. Lymphocytes were incubated at a concentration of 10^7 cells/ml in MEM with antibiotics and without serum for 22 hours. The viability of the lymphocytes was tested by trypan blue exclusion staining and less than 5% of the cells were found dead. After centrifugation at $1000 \times g$ for 10 min, the supernatant fluid was incubated with labelled *B. cereus* DNA at a concentration of 100 μg/ml. Samples were taken after 6.5 and 24 hours of incubation and analyzed by agarose-gel electrophoresis. The data in Fig. 4 indicate that

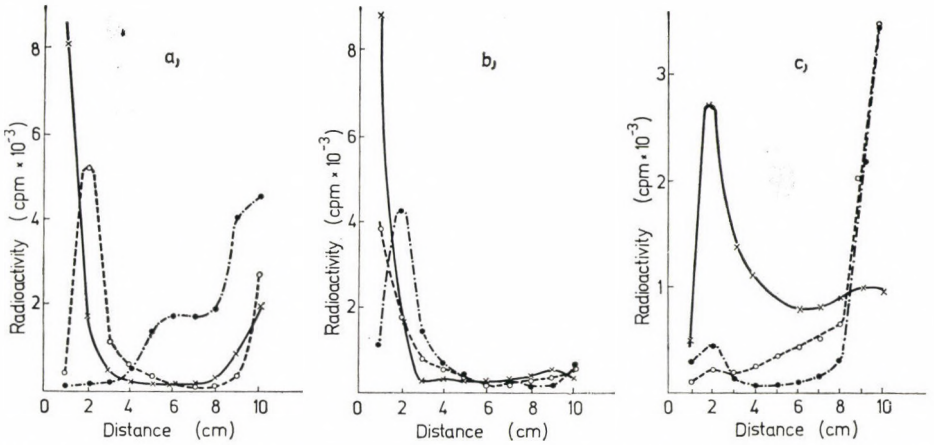

Fig. 3. Agarose-gel electrophoresis of DNA after different times of incubation with a cellular extract prepared as described in Materials and methods. a) Enzyme activity at pH 4.5. Incubation times: × — ×, 5 min; ○ - - - ○, 20 min; ● . - . - . ●, 60 min. b) Enzyme activity at pH 7.4. Incubation time: × — ×, 0 min; ○ - - - ○, 60 min; ● . - . - ●, 5 hrs. c) Enzyme activity at pH 8.5. Incubation time: × — ×, 0 min; ○ - - - ○, 30 min; ● . - . - . ●, 180 min

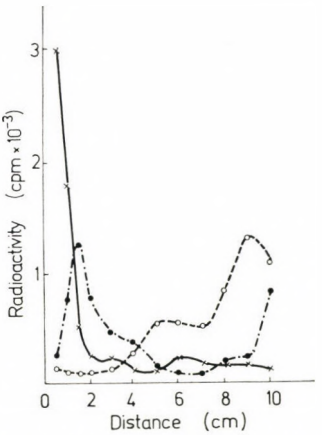

Fig. 4. Agarose-gel electrophoresis of 100 μg of labelled DNA incubated with 1 ml of the supernatant from 10^7 cells incubated for 22 hours at 37 °C. DNA pattern after an incubation time of 0 hour (× — ×), 6.5 hours (● — ●) and 22 hours (○ — ○)

the peak of highly polymerized DNA diminished at 6.5 hours and disappeared at 22 hours. Small DNA molecules began to appear at 6.5 hours but after 24 hours of incubation radioactivity was found only in the terminal portion of the gel.

Discussion

During the past 25 years evidence for the uptake of DNA molecules by living mammalian cells has been accumulating. Most of the DNA uptake tests were carried out for only a few hours (Bhargava, Shanmugam, 1971). In the experiments described in this work DNA uptake was followed for 22 hours and the amount of DNA was measured both in the cells and in the incubation medium as acid-soluble and acid-precipitable material. According to our observations the uptake of DNA by lymphocytes follows a biphasic curve. These data are in fair agreement with the experiments described by Groneberg et al. (1975) concerning the uptake of adenovirus by KB cells. On the other hand, with murine lymphocyte cultures (Ayad, Fox, 1968; Robins, Taylor, 1968) uptake of homologous DNA was linear even upon prolonged incubation.

Electrophoretic analyses suggested that in the cells which had taken up highly polymerized DNA molecules the foreign DNA was degraded by endonucleases.

The observed two phases of uptake may serve as a model to suggest that in the first few hours the cells do take up high-molecular weight DNA but this event induces an expelling process. The fact that in the second phase of uptake mainly smaller DNA fragments can be detected in the incubation medium suggests a new oligonucleotide reutilization process, in spite of the generally accepted idea that low-molecular weight DNA is insufficiently taken up by mammalian cells (Bhargava, Shanmugam, 1971).

No difference was found in the pattern of the saturation curves obtained with cells after 2 hours of incubation when most of the DNA was highly polymerized and with cells after 22 hours of incubation when most of the DNA was degraded. These findings suggest that under our experimental conditions DNA uptake did not depend on the size of the DNA molecules and was affected only by the dose of DNA.

Literature data on the uptake of native or heat denatured DNA by eukaryotic cells are ambiguous (Bhargava, Shanmugam, 1971). We have found that the uptake of heterologous DNA by lymphocytes does not necessarily require the native form of DNA. This is in agreement with the finding of Keprtová (1974).

In uptake experiments it is proper to consider the role of DNases, both in the cells and in the incubation medium. For this reason serum has been omitted from the cell culture medium.

Progressive DNA size reduction was reported in the uptake of T7 DNA by lymphoblasts (Kao et al., 1973) and in that of adenovirus DNA by KB cells (Groneberg et al., 1975). In our experiments the amount of acid-soluble DNA was very low during the first few hours, in agreement with other observations (Bhargava, Shanmugam, 1971), and even later this values did not change significantly. In spite of that, our electrophoretic patterns showed progressive DNA size reduction in the acid-insoluble fraction upon prolonged incubation. This observation was true for the foreign DNA both inside the cell and in the incuba-

tion medium, suggesting that cell nucleases were released into the culture medium by living cells. The facts that the supernatants of 22 hours old cell cultures were capable of degrading *B. cereus* DNA similarly to the cells and that no more than 5% of the lymphocytes were found dead after 22 hours of incubation supported this finding.

The appearance of degraded DNA molecules in the incubation medium shows that DNA is taken up by the cells from a pool of polynucleotides of decreasing molecular weight.

References

Ayad, S. R., Fox, M. (1968) Nature *220* 35
Bhargava, P. M., Shanmugam, G. (1971) Progr. Nucleic Acid Res. and Mol. Biol. *11* 103
Csuzi, S. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. *6* 377
Eagle, H. (1959) Science *130* 432
Groneberg, J., Brown, D. T., Doerfler, W. (1975) Virology *64* 115
Helling, R. B., Goodman, H. M., Boyer, H. W. (1974) J. Virol. *14* 1235
Kaol, P. C., Regan, J. D., Volkin, E. (1973) Biochim. Biophys. Acta *324* 1
Keprtová, J. (1974) Studia Biophysica *42* 111
Laskowski, M. (1967) Advances in Enzymol. *29* 167
Ledoux, L. (1965) Progr. Nucleic Acid Res. and Mol. Biol. *4* 231
Meizel, S., Kay, R. M. (1964) Biochim. Biophys. Acta *103* 431
Natani, N. K., Setlow, J. K. (1974) Progr. Nucleic Acid Res. and Mol. Biol. *14* 39
Piffko, P., Köteles, G. J., Antoni, F. (1970) Pract. Oto-rhino-laryng. *32* 350
Robins, A. A., Taylor, D. M. (1968) Nature *217* 1228
Saito, H., Miura, K. (1963) Biochim. Biophys. Acta *72* 619
Staub, M. (1974) Abstracts 9th FEBS Meeting, p. 163
Zölner, E. J., Helm, W., Zahn, K. R., Reitz, M. (1974) Nucleic Acid Res. *1* 1069

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Ultrastructural, Optical and Volume Changes in Brain Mitochondria During Gramicidin-induced Ion Uptake

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Gramicidin-induced swelling of rat brain mitochondria was followed spectrophotometrically with a parallel analysis of changes in the ultrastructure, volume and eccentricity ratio of the mitochondria. Under the effect of gramicidin the expansion of the inner compartment occurs with an overall increase in volume. Mitochondria tend to be rounded although not entirely spherical in shape. The reversal of swelling with respiratory inhibitors (KCN, rotenone) results in a shrinkage of the inner compartment and is not accompanied by a proportional reduction of the outer compartment. Mitochondria fail to regain their original shape. It can be concluded that in the course of energy-dependent swelling the ability to contract might be retained, whereas other factors controlling the shape of the mitochondrion are irreversibly affected.

Introduction

Gramicidin induces respiration-dependent uptake of monovalent cations through the mitochondrial inner membrane followed by swelling (Chappell, Crofts, 1965; Moore, 1968). The morphology of the swelling process in brain mitochondria has scarcely been studied (Minn et al., 1975). The purpose of the present study was to correlate changes in apparent optical density with alterations in the ultrastructure, volume and shape of brain mitochondria.

Materials and methods

Rat brain mitochondria were isolated by the method of Clark and Nicklas (1970). The fractions were occasionally checked for controlled respiration in a Gilson oxygraph. Respiratory control ratios were between 3 and 6 with glutamate *plus* malate or pyruvate *plus* malate as substrates. Optical density changes were measured at 520 nm using a Spektromom 202 type spectrophotometer equipped with a recorder. The measurements were carried out at room temperature. At the maximum of optical density change the content of the cuvette was transferred to a centrifuge tube containing 3 ml of Karnovsky's fixative. Samples from two parallel experiments (3 ml each) were added to the fixative and fixed for

60 min. Then mitochondria were pelleted, washed in phosphate buffer for at least 60 min and postfixed with 1% osmic acid for another 60 min. Further processing and embedding in Durcupan (Fluka) was carried out according to Hajós (1975). Ultra-thin sections were cut with a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, viewed and photographed with a Tesla BS 413 A electron microscope. Protein was determined by the biuret method. Gramicidin D was obtained from Serva. All other reagents were of the highest purity available.

Results and discussion

Rat brain mitochondria oxidizing pyruvate *plus* malate (state 4) showed a rapid swelling on addition of 5×10^{-6} M gramicidin (Fig. 1a), accompanied by a decrease in optical density. Subsequently added KCN or rotenone increased optical density to a level usually even higher than that observed in state 4. In isotonic medium the amplitude of swelling was low, therefore, hypotonic media were used (Falcone, Hadler, 1968). In addition, EDTA was needed to obtain maximal swelling and a complete reversal of the swelling with respiratory inhibitors.

Changes in light scattering were accompanied by marked changes in the ultrastructure of mitochondria. Unincubated mitochondria had highly electron-dense matrices with deep lamellar infoldings of the inner membrane (Fig. 1b). Occasionally, bulges of the outer membrane could be seen. Fig. 1c shows state 4 mitochondria incubated in an EDTA-containing medium. No gross change in the structure can be seen. At the maximum of gramicidin-induced swelling (Fig. 1d) mitochondria were rounded, with light matrix. Inner membrane invaginations, although reduced in number and size, could still be recognized. Some of the mitochondria, however, remained unaltered even at the maximum of turbidity response.

Table 1

Volumes of brain mitochondria in the course of gramicidin-induced swelling and reversal of swelling with KCN

The longest and the short perpendicular diameters of 180 to 300 mitochondrial profiles for each given condition were measured and volumes were calculated using the formula $ab^2\pi/6$, where a and b represent the longest and the short perpendicular diameters, respectively. To diminish errors originating from tangential sectioning mean volumes were calculated from the upper 10 per cent of the values (figures in brackets). Values are expressed as the mean $\pm$ standard error, in μ^3

	Condition			
	Control	EDTA-containing medium	EDTA-containing medium + gramicidin	EDTA-containing medium + gramicidin + KCN
Volume	0.12 ± 0.008 (30)	0.10 ± 0.005 (31)	0.20 ± 0.013 (19)	0.11 ± 0.003 (26)

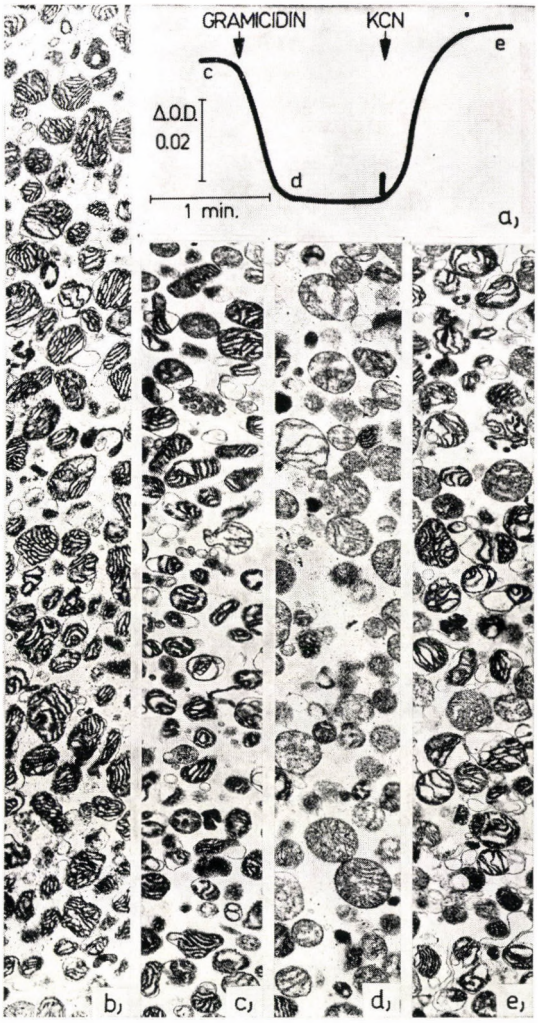

Fig. 1. Correlation between light scattering changes and mitochondrial structure in the course of a typical swelling cycle following the subsequent addition of 5×10^{-6} M gramicidin and 0.6 mM KCN to mitochondria incubated in a medium of 125 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-phosphate (pH 7.4), 4 mM Tris-pyruvate, 2 mM Tris-malate and 1 mM K-EDTA. 0.6 mg mitochondrial protein in 3 ml final volume. Samples for electron microscopy were taken where indicated on the curve

On addition of KCN (Fig. 1e) or rotenone the mitochondria did not fully regain the structure observed prior to swelling in spite of the virtually complete reversal of gramicidin-induced optical density change. There was a marked increase in the number of mitochondria with dark matrix as compared to Fig. 1d, but disorientation of the inner membrane lamellae could be observed and the outer compart-

Table 2

Distribution of eccentricity ratios of mitochondrial diameters in the course of gramicidin-induced swelling

p values indicating the levels of statistical significance of the deviation from the control were obtained by a homogeneity test (chi²-test). Fields containing less than five elements were additively evaluated

Eccentricity	1.0—1.2	1.2—1.4	1.4—1.6	1.6—1.8	1.8—2.0	2.0—2.2	2.2—2.4	2.4—2.6	2.6<	
	Number of mitochondrial profiles									Total
Control	62	85	37	43	27	15	12	7	11	299
EDTA-containing medium*	52	94	54	35	26	11	6	8	11	297
EDTA-containing medium+gramicidin**	22	89	36	14	9	4	1	1	4	180
EDTA-containing medium+gramicidin+KCN**	76	104	43	14	15	2	1	0	4	259

* $p < 0.05$
** $p < 0.01$

ment remained expanded. In a considerable number of mitochondria the inner compartment remained moderately swollen.

The results of volume measurements are shown in Table 1. There is a substantial increase in the mean volume of mitochondria treated with gramicidin, while the addition of KCN apparently reverses this change. This corresponds to the disappearance of the most extensively swollen mitochondrial forms. It is of interest that the mean volume of mitochondria incubated in a respiratory medium with EDTA does not differ greatly from that of the control, supporting the morphological observation that brain mitochondria may retain a condensed form during state 4 respiration. Expanded mitochondria or a tendency towards expansion were characteristic of aged or impaired fractions. Thus, in brain mitochondria, the expansion of the matrix cannot be regarded as specific for state 4 respiration. Similar conclusions were drawn by Sordahl et al. (1969) in heart mitochondria, but this was strongly disputed by Hatase et al. (1971). Following KCN treatment, light scattering usually exceeded that of the initial state (phase "e" of the curve in Fig. 1), whereas no such "overshoot" was seen in the mean volume. This could be attributed to a condensation of the matrix without a proportional decrease in volume of the outer compartment. The estimation of volume may be inadequate

or too much simplified where the mitochondria possess elongated or entirely irregular shapes. For an approximate calculation, however, it was postulated that isolated mitochondria are generally ellipsoid bodies.

The distribution of eccentricity ratios of mitochondrial diameters shows that under the effect of gramicidin the values were strongly shifted towards the lower ranges, for the bulk of the mitochondria they fell between 1.2 and 1.4 (Table 2). This means that the majority of swollen mitochondria tended to be of a more rounded although not entirely spherical shape. The reversal of swelling with cyanide did not seem to be a complete return to the initial forms; the mitochondria retained their rounded shape. This suggests that in this type of mitochondrial swelling the ability to contract might be retained whereas other factors controlling the shape of the mitochondrion are irreversibly affected.

References

Chappell, J. B., Crofts, A. R. (1965) Biochem. J. *95* 393—402
Clark, J. B., Nicklas, W. J. (1970) J. Biol. Chem. *245* 4724—4734
Falcone, A. B., Hadler, H. I. (1968) Arch. Biochem. Biophys. *124* 91—109
Hajós, F. (1975) Brain Res. *93* 485—489
Hatase, O., Wakabayashi, T., Green, D. E. (1971) J. Bioenergetics *2* 183—195
Minn, A., Gayet, J., Delorme, P. (1975) J. Neurochem. *24* 149—156
Moore, C. L. (1968) J. Neurochem. *15* 883—902
Sordahl, L. A., Blailock, Z. R., Kraft, G. H., Schwartz, A. (1969) Arch. Biochem. Biophys. *132* 404—415

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Investigation of Peptides Containing Half-cystine in Pig Pancreas Amylase

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Sequence analysis of the carboxymethyl-cystein-containing tryptic peptides isolated after the total reduction and carboxymethylation of pig pancreas amylase has shown that the half-cystine-containing tryptic peptides of the isozymes have identical sequences. The fact that 10 tryptic peptides containing carboxymethyl-cystein could be isolated, supports the generally accepted view that pig pancreas amylase contains single polypeptide chain.

Pig pancreas amylase is a protein of a molecular weight of 50–52 000 daltons. It consists of a single polypeptide chain (Cozzone et al., 1970; Závodszky, Elődi, 1970) and contains two thiol groups and four disulphide bridges per molecule (Schramm, 1964; Straub et al., 1970).

This study was done to compare the half-cystine-containing peptides of the isozymes and to determine their sequences.

Materials and methods

Pig pancreas amylase was prepared according to Hatfaludi and Straub (1966) and recrystallized three times. The isozymes were prepared by ion exchange on a DEAE-Sephadex column according to the method of Szabó and Straub (1966).

To carry out total reduction and carboxymethylation, the protein was dissolved in 8 M urea, containing 40 mM phosphate buffer and 5 mM EDTA (pH 8.5), to a final half-cystine concentration of 2 mM. The molecular weight was taken as 50 000 *D*. Reduction was carried out with dithioerithritol (2.5 moles per mole of half-cystine) at room temperature for 2 hours, then [^{14}C]-bromoacetate (Isotope Institute, Budapest; 2.4 mCi/mmole) was added (5 moles per mole of total SH), and the mixture was carboxymethylated for two hours. Excess bromoacetate was removed by the addition of a calculated amount of dithioerithritol. The mixture was then dialyzed against 1 mM hydrochloric acid to remove salts.

Tryptic hydrolysis was carried out with trypsin (Calbiochem, essentially free of chymotrypsin) at a trypsin: protein ratio of 1 : 40, in 100 mM ammonium-bicarbonate (pH 8.0) at 37 °C for 4 hours.

The tryptic hydrolysate was first separated on a Sephadex G 25 (fine) column. Three well-separated radioactive peaks appeared (Fig. 1).

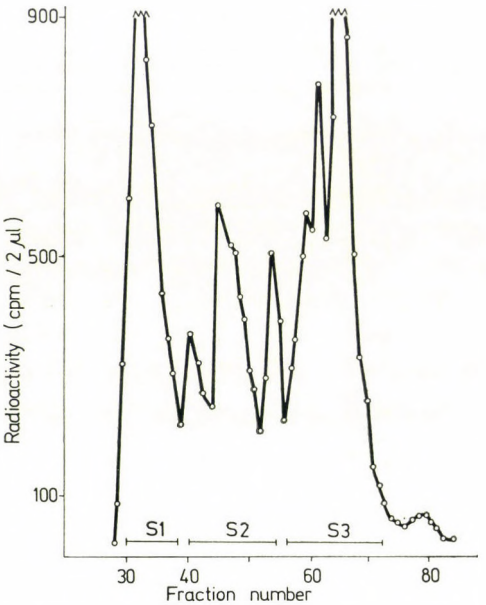

Fig. 1. Gel-filtration of the tryptic hydrolysate of reduced and fully carboxymethylated amylase on a Sephadex G—25 (fine) column equilibrated with 0.1 M ammonium bicarbonate. Size of column: 180×2 cm; flow rate: 25 ml/hour; eluent: 0.1 M ammonium bicarbonate; V_0=370 ml; fractions taken at 12 min intervals

The individual peptides present in the radioactive peaks were isolated by paper electrophoresis and paper chromatography. The location of the radioactive peptides was determined by radioautography (highly sensitive X-ray film, Forte, Vác, Hungary), applying an exposition time of 24 to 28 hours.

The sequences of the isolated radioactive peptides were determined by the dansyl-Edman method (Hartley, 1970) the dansyl-amino acids were identified by thin-layer electrophoresis (Sajgó, 1970) and thin-layer chromatography (Hartley, 1970). C-terminal sequences were determined by a micro-method (Sajgó, Dévényi, 1972) involving digestion of peptides with carboxypeptidase and identification of the cleaved amino acids by thin-layer ion exchange (Dévényi et al., 1971). The resin-coated chromatoplates applied were IONEX-25 (Macherey-Nagel and Co., Düren) and FIXION 50 (Chinoin, Nagytétény–Budapest, Hungary).

Results and discussion

First, we compared the fingerprints and the radioautograms of the tryptic hydrolysates obtained after the total reduction and carboxymethylation of the isolated amylase isozymes. The location of each radioactive peptide on the fingerprints of the two isozymes was identical (Fig. 2A): they fully overlapped. Therefore

only the radioautogram of amylase I is shown in Fig. 2A. This observation indicates that even if there are differences in the sequences of the isozymes of pig pancreas amylase, this cannot be due to structural heterogeneity of the tryptic peptides containing half-cystine. Therefore, we felt safe to use a mixture of isozymes for the isolation of the peptides containing half-cystine.

Ten radioactive peptides containing carboxymethyl-cystein were isolated from the tryptic hydrolysate of pig pancreas amylase, as seen in Fig. 2B, C and D. The total sequences of peptides 1, 2, 3, 4, 6 and 7 were determined (Table 1).

Peptide 1 shows the behaviour of a basic peptide upon electrophoresis at pH 6.5. This is in contradiction to the results of the amino acid analysis and sequence determination (Table 1). At the present time we have no experimental explanation for this observation.

Table 1

Structure of the carboxymethyl-cystein containing tryptic peptides isolated from pig pancreas amylase

The peptides were hydrolysed in N_2 atmosphere with 5.7 N HCl, at 105 °C, for 24 hours, the amino acid analysis was carried out by a single column rapid method (Dévényi, 1969) in a BIOCAL BC 200 amino acid analyzer. Results are expressed in nanomole. The sequences set in ïtalics were determined by carboxypeptidase method

Peptide number*	Amino acid composition (nanomoles)	Sequence
1	arg 80; cmc 50; ser 150; val 70; tyr: 50;	val-ser-ser-ser-cmc-*tyr-arg*
2	arg 80; cmc 50; thr 120; leu 70	leu-cmc-thr-thr-arg
3	lys 220; cmc 130	cmc-lys
4	arg 60; cmc 70; asp 140; gly 80; val 150	cmc-asx-val-gly-*val-arg*
5	lys 200; cmc 150; asp 270; thr 190; ser 190; gly 320; val 220; ile 200	val-gly-asx/$cmc_1thr_1ser_1gly_{0-1}$/ -*ile-lys*
6	arg 90; cmc 30; asp 110; thr 70; val 80	cmc-asx-val-*thr-arg*
7	lys 80; cmc 20; asp 80; ser 60; gly 70; val 30; ile 40	cmc-asx-val-ile-ser-gly-lys
8	arg 30; cmc 30; asp 60; thr 50; ser 30; gly 120; ala 50; leu 20; tyr +	asx-/$cmc_1asx_1thr_{1-2}ser_1gly_5$ ala_2trp_x/-*tyr-leu-arg*
9	lys 20; cmc 10; his 10; tyr +; ile 20; leu 20; val 10; gly 15; ala 15; thr 20; ser 20; asp 35	gly-/$cmc_1his_1tyr_xile_{1-2}leu_{1-2}$ $val_{0-1}gly_1ala_1thr_1ser_1asx_2$/-*lys*
10	lys 70; cmc 30; asp 110; glu 50; ala 60; val 20; leu 40	ala-/$cmc_1asx_2glx_1val_1leu_1$/-lys
10 P**	asp 60; glu 30; ala 40; cmc +	ala-asx-/$cmc_1glx_1asx_1$/

* See Fig. 2A

** Peptic fragment of peptide 10

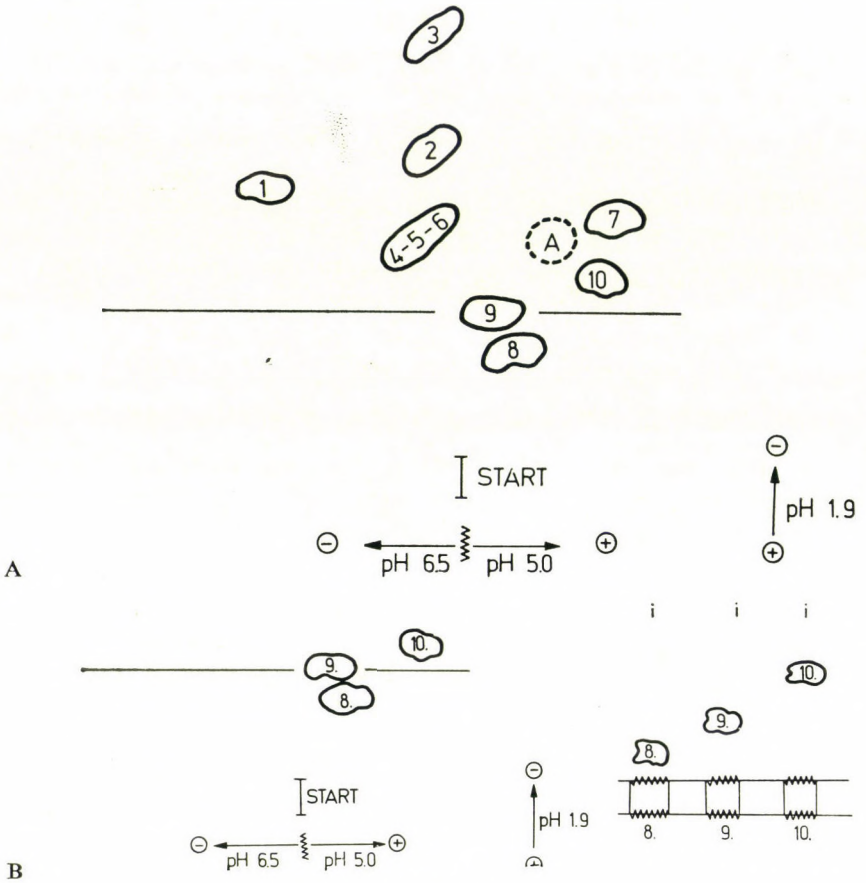

Fig. 2A—B

Each peptide, on the basis of its C-terminal residue, seems to be a product of tryptic cleavage. This fact, however, does not exclude the possibility of chymotryptic miscleavage. For instance, supposing the C-terminal sequence in peptide 9 is tyr- tyr-cmc-lys, peptide 3 could be a derivative of this terminal. Sequence analyses of peptides containing thiol groups of amylase and partially reduced amylase (Telegdi et al., 1976) indicated that the thiol groups in peptides 3 and 9 can be labelled with [^{14}C]-bromoacetate independently of each other. Thus, homology due to chymotryptic miscleavage of the two peptides can be excluded. Similarly, peptide 5 might be a derivative of peptide 9. This possibility is, however, excluded by the C-terminal sequences of the peptides in question; while it was . . . ile-lys in peptide 5, under similar conditions the cleavage of peptide 9 stopped after the C-terminal lys residue. It follows from the specificities of the carboxypeptidases (Ambler, 1967) that the sequences of peptides 5 and 9 are different.

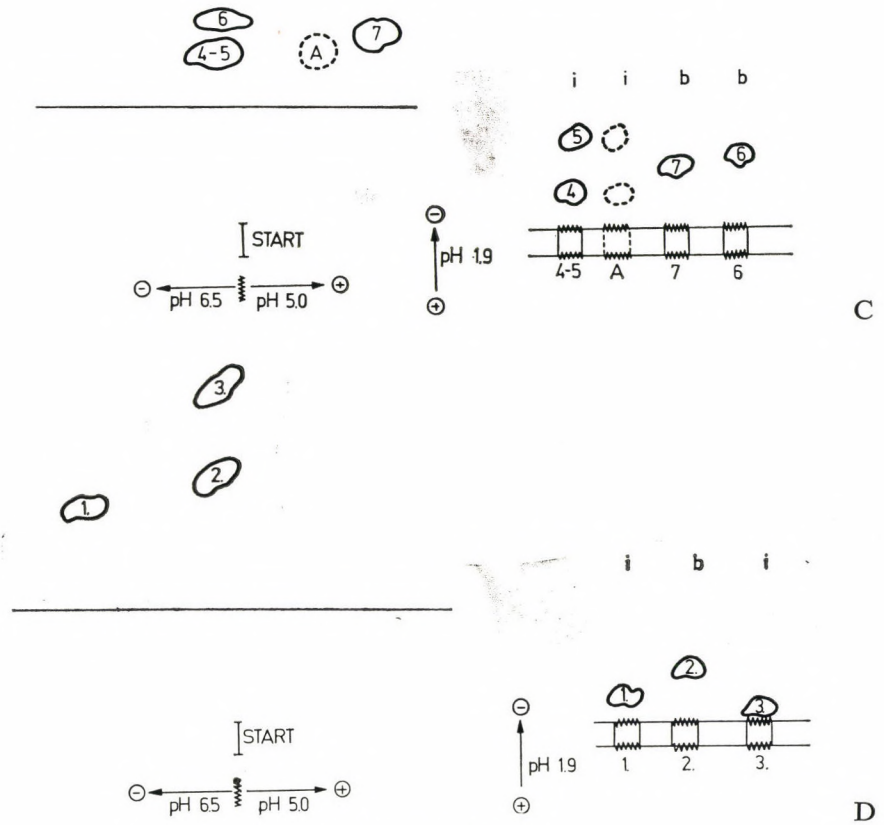

Fig. 2 C—D

Fig. 2. Position and preparation of carboxymethyl-cysteine containing peptides of the tryptic digest of fully carboxymethylated amylase. Carboxymethylation was performed with [^{14}C]-bromoacetate. A: Radioautogram of the fingerprint of the tryptic hydrolysate of the isolated isozymes (see text). B—D: Isolation of the radioactive peptides of radioactive peaks S1 (B), S2 (C) and S3 (D) isolated by gel-filtration of the tryptic hydrolysate, respectively

Paper electrophoresis in the first dimension was performed in a double-buffer system (Dévényi 1963), with buffer pH 5 at the cathodic side, with buffer pH 6.5 at the anodic side. In the second dimension electrophoresis was performed at pH 1.9. The horizontal line shows the mobility of the control ε-DNP-lysin, at pH 1.9

Paper chromatography was performed in the "third" dimension: "i": ascending chromatography in pyridine : isoamylalcohol : water = 35 : 35 : 30 (v/v). "b": ascending chromatography in butanol : glacial acetic acid : water = 120 : 30 : 50 (v/v) systems. Other details see: Fábián (1970)

As described above, we have isolated 10 carboxymethyl-cysteinyl peptides of different sequences from the tryptic hydrolysate of amylase. The same number was suggested by amino acid analyses (Straub et al., 1970) and the fact that the protein consists of a single chain (Závodszky, Elődi, 1970; Cozzone et al., 1970) also supports the correctness of the above figure.

Analysis of the radioactive peptides from peak S 2 showed that peptide A in the total hydrolysate is not homogeneous, it can be separated by chromatography into two peptides of sequences identical with those of peptides 4 and 5 (Fig. 2C). Considering the electrophoretic and chromatographic mobility of peptides "A" and 5, we suggest that this phenomenon is due to deamination of peptides 4 and 5. Yield analysis of the peptides suggested that about 40 per cent of both peptides were present in deaminated form. Since deamination occurred in both isozymes (Fig. 2A), the results of Cozzone et al. (1970) concerning differences in the amide content of the isozymes could not be supported at least as far as the analysis of cysteinyl peptides in concerned. It is difficult to test experimentally whether deamination occurs also *in vivo* or, else, only during the isolation of the peptide. The fact, however, that until the fingerprints of the isozymes are prepared the protein is exposed to relatively mild conditions (see Materials and methods) makes the former alternative more probable.

The sequences of the thiol-containing peptides of the isolated isozymes and the analytical results concerning partially reduced amylase will be published elsewhere (Telegdi et al., 1976).

References

Ambler, R. P. (1967), in Methods in Enzymology, Vol. XI. C. H. Hirs (ed.). Acad. Press London p. 155

Cozzone, Pasero, P., Marchis-Mauren, G., (1970) Biochim. Biophys. Acta *200* 590

Dévényi, T. (1963) Magyar Kémiai Folyóirat *69* 538

Dévényi, T. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 297

Dévényi, T., Hazai, I., Ferenczi S., Báti, J. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. *6* 3851

Fábián, F. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 445

Hartley, B. S. (1970) Biochem. J. *119* 805

Hatfaludy, F., Strashilov, T., Straub, F. B. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 39

Sajgó, M. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 231

Sajgó, M., Dévényi, T. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. *7* 233

Schramm, M. (1964) Biochemistry *3* 1231

Straub, F. B., Szabó, M., Dévényi, T. (1970) in Enzymes and Isoenzymes. Acad. Press, London, p. 257

Szabó, M., Straub, F. B. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 379

Telegdi, M., Fábián, F., Shehata M. El-Sewedy, Straub, F. B. (1976) Biochim. Biophys. Acta *429* 860

Závodszky, P., Elődi, P. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 225

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^{18}O-Exchange Catalyzed by Myosin, Heavy Meromyosin, Heavy Meromyosin Subfragment 1 and their Complexes with Actin

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Myosin, HMM and HMM S1 catalyze ^{18}O-exchange between P_i and $H_2{}^{18}O$ of the medium at an intermediate stage of ATP hydrolysis ("intermediate ^{18}O-exchange") in the presence of Mg^{2+}. Natural complexes of actomyosin and acto-HMM S1 do not catalyze intermediate ^{18}O-exchange but facilitate "direct" or "medium" ^{18}O-exchange ($KH_2P^{18}O_4 \rightleftharpoons H_2O$) even without ATP. Reconstituted complexes of acto-myosin, acto-HMM, acto-HMM S1, PABC-HMM S1, congo-myosin and TNP-myosin do not catalyze direct ^{18}O-exchange in the presence of Mg^{2+} and absence of ATP. From the data obtained a hypothetical sequence of phosphorylation and ^{18}O-exchange reactions in myofibril action has been suggested.

Introduction

The mechanism of the transformation of the chemical energy of ATP into muscle work is so far unclear. Some hypotheses claim that the enzymatic hydrolysis of ATP by myosin consists of many steps. One of the steps may be an intermediate phosphorylation of myosin at the expense of ATP or a noncovalent binding of ATP γ-terminal phosphate to myosin (Boyer, 1967; Bagshaw, Trentham, 1973). Evidence for the latter hypothesis has been presented when ATP hydrolysis by myosin was carried out in a medium enriched by $H_2{}^{18}O$ and an oxygen isotope exchange was found to occur between P_i released from ATP and $H_2{}^{18}O$ of the medium (Levy, Koshland, 1959). As it became clear later, the incorporation of ^{18}O from $H_2{}^{18}O$ into P_i in the course of hydrolysis was the result of two distinct types of ^{18}O-exchange reactions. These are the "intermediate exchange" reflecting an intermediate phosphorylation step during ATP hydrolysis and the "medium" or "direct" exchange occurring with the P_i of the medium (Koshland, Levy, 1964; Boyer, 1964; Panteleeva, 1969). The difference between the two reactions is that whereas the extent of medium exchange depends on incubation time, enzyme and P_i concentrations, these factors do not influence intermediate exchange. The latter

Abbreviations used: HMM, heavy meromyosin; HMM S1, subfragment 1 of heavy meromyosin; PABC-HMM S1, paraaminobenzenecellulose-HMM S1; TBS, 2,4,6-trinitrobenzenesulfonate; TNP-myosin, trinitrophenylated myosin.

exchange was found to involve up to 3 atoms ^{18}O per P_i molecule (in addition to 1 atom ^{18}O incorporated due to hydrolytic addition of ^{18}OH from $H^{18}OH$), while medium exchange involved as many as 4 atoms ^{18}O per P_i molecule.

Both reactions are catalyzed not only by myosin, but also by its catalytically active fragments, i.e. heavy meromyosin (Yount, Koshland, 1963; Swanson, Yount, 1966) and heavy meromyosin subfragment 1 (Panteleeva et al., 1972). Therefore ^{18}O-exchange can be considered as a property of the active site of myosin ATPase.

In this paper the influence of actin, a natural modificator of myosin ATPase on ^{18}O-exchange catalyzed by myosin, HMM and HMM Sl is presented. ^{18}O-exchange is studied here with special regard to muscle protein interactions occurring in muscle contraction. ^{18}O-exchange reactions in the actomyosin ATPase system are compared with those in the Na^+-K^+-dependent membrane ATPase system.

Materials and methods

Myosin, actin and natural actomyosin were prepared from rabbit skeletal muscle (Ivanov, Yuryev, 1961). HMM was prepared by tryptic digestion (Szent-Györgyi, 1953) and HMM Sl by papain digestion according to Lowey et al. (1969) or by tryptic digestion of actomyosin in the presence of EDTA, according to Biró et al. (1973). The latter method was used also for preparing "natural" acto-HMM Sl complex. Reconstituted complexes of actin with myosin, HMM and HMM Sl were made by mixing actin with these proteins in weight ratios of 1 : 1, 1 : 2 and 1 : 3, respectively.

Trinitrophenylated myosin was prepared by treating myosin with TBS according to Fábián and Mühlrad (1968). Paraaminobenzenecellulose-HMM Sl complex was made by the treatment of HMM Sl with PABC (Liu-Osheroff, Guillory, 1972).

Upon addition of 30 μg of congo-red dye per 1 mg of myosin in 0.25 M KCl complex congo-myosin was formed (Ashmarin, 1951).

Na^+-K^+-dependent ATPase was obtained in the form of membranes from the kidney cortex of guinea pigs according to Post and Sen (1967) with Pisareva's modifications (1968).

ATPase and ^{18}O-exchange activities of myofibrillar proteins were determined in the following medium: 0.15 M tris-acetate buffer, pH 7.4; 0.1 M KCl, 0.01 M $MgCl_2$, 0.005 or 0.01 M ATP; myosin 1–2, or HMM 0.5, or HMM Sl 0.2–0.5 mg · ml^{-1}; incubation was carried out at 25°. ATPase and ^{18}O-exchange activities of the membranes were determined under conditions optimal for ^{18}O-exchange (Skvorstevich et al., 1972).

To estimate the extent of intermediate and medium ^{18}O-exchange during ATP hydrolysis two types of test were performed with the same preparation of ATPase. The first test was hydrolysis of ATP (0.005 M) in $H_2{}^{18}O$ (1 atom % excess

of ^{18}O) in the presence of unlabelled P_i (0.005 M). The second test was hydrolysis of ATP (0.005 M) in unlabelled H_2O in the presence of P_i (0.005 M) labelled by ^{18}O. The reaction mixtures were incubated until 100% hydrolysis was attained (usually 4–6 hours).

In the first test ^{18}O was incorporated into P_i mainly as a result of intermediate exchange; in the second one there was a loss of the label from P_i as a result of medium exchange.

A comparison of the results of the two tests (taking into consideration O^{18}-label dilution) makes it possible to calculate the extent of net intermediate exchange (Levy et al., 1962).

In other experiments medium exchange was tested in the absence of ATP hydrolysis. The incubation mixture contained either 0.01 M $KH_2P^{18}O_4$ (1 atom % excess of ^{18}O), 0.005 M ADP and 0.005 M $MnCl_2$ or 0.018 M $KH_2P^{18}O_4$ and 0.01 M $MgCl_2$ (Ilin, Panteleeva, 1967). Incubation was for 24 hours at 25°.

P_i (added or released from ATP as a result of hydrolysis) was separated as barium salt and was analysed for ^{18}O content by mass-spectrometry. Before mass-spectrometry the oxygen of barium phosphate and that of H_2O (the medium for ^{18}O-exchange) were transformed to CO_2 by reaction with guanidine hydrochloride (Boyer et al., 1961) according to Ilin (1966).

Values of the isotope exchange were expressed in terms of ^{18}O atoms per P_i molecule.

P_i concentrations were determined according to Fiske and Subba Row (1925). Protein content was estimated by a microbiuret method (Itzhaki, Gill, 1964).

Results and discussion

The capacity to catalyze intermediate ^{18}O-exchange in the process of ATP hydrolysis is a property of the active site of myosin ATPase. As can be seen from Table 1, purified preparations of enzymatically active fragments of myosin, HMM and HMM S1 catalyze the intermediate ^{18}O-exchange to the same extent as original myosin does (about 2.1–2.8 atoms per P_i molecule).

Upon the addition of actin to myosin, HMM and HMM S1 (reconstituted complexes) the intermediate exchange decreases to 1.0–1.5 atoms ^{18}O per P_i molecule (Table 1). The level of medium exchange remains nearly the same or is slightly higher than with pure HMM and HMM S1 (Table 1), i.e. 0.3–0.6 atoms ^{18}O per P_i molecule.

Natural actomyosin and acto-HMM S1 prepared from natural actomyosin practically display no intermediate ^{18}O-exchange in the presence of Mg^{2+}. The medium exchange observed in these experiments is also small (Table 1). Thus the extent of ^{18}O-exchange catalyzed by "natural" and reconstituted actin complexes is distinctly different.

Another feature of natural complexes as compared with reconstituted ones lies in their ability to catalyze medium ^{18}O-exchange in the absence ATP or ADP,

Table 1

Intermediate and medium ^{18}O-exchange reactions catalyzed by reconstituted and natural rabbit actomyosin, acto-HMM and acto-HMM S1 in the presence of $Mg^{2}{}_{+}$ in the course of ATP hydrolysis

Perparation	ATPase activity μmole P_i $mg^{-1} \times min^{-1}$	Number of ^{18}O atoms per P_i molecule*	
		Intermediate exchange	Medium exchange
Myosin	0.01	2.84	0
Myosin + actin	0.05	0.95	0.61
Natural actomyosin	0.10	0.10	0.26
HMM	0.03	2.09	0.52
HMM + actin	0.03	1.46	0.34
HMM S1	0.08	2.50	0
HMM S1 + actin	0.22	1.34	0.60
"Natural" acto-HMM S1	0.12	0.20	0.18

* ^{18}O incorporated in addition to 1.0 atom expected from cleavage due to hydrolysis

The extent of the reaction depends on protein concentration (Table 2). Myosin, its proteolytic fragments and reconstituted complexes do not catalyze medium exchange in the absence of ADP or ATP, though the ATPase activity of the complexes is increased as compared with original myosin and HMM S1 (Table 1).

Thus, binding of actin to myosin and HMM S1 in "natural" complexes (possibly containing some additional factors as compared with reconstituted purified complexes) radically transforms the process of isotope oxygen exchange: intermediate exchange is replaced by medium exchange.

Some modifications of myosin have a similar activating effect on its Mg^{2+}-ATPase activity as the complex formation with actin. We tested the influence of a few modifications of this kind (trinitrophenylation and complexing with PABC) on medium ^{18}O-exchange.

TBS-treatment results in a 6-fold activation of ATPase whereas treatment of HMM S1 with PABC increases enzyme activity by a factor of more than two. These results are consistent with available data (Fábián, Mühlrad, 1968; Liu-Osheroff, Guillory, 1972).

TBS treatment and complexing with PABC did not enhance medium exchange. The enzyme behaved thus similarly to that activited by complexing with actin (Table 3). Mixing of myosin with the dye congo-red resulting in "superprecipitating" complex formation (Ashmarin, 1951) does not induce any extra ^{18}O-exchange either (Table 3). The modificators do not influence the exchange reaction observed under optimal conditions for medium exchange in the presence of ADP and Mn^{2+} either.

Table 2

Medium ^{18}O-exchange reaction catalyzed by natural and reconstituted complexes of actin with myosin and HMM S1 in the absence of ATP

Preparation	Protein concentration mg × ml^{-1}	Number of ^{18}O atoms per P_i molecule
Natural actomyosin	1.4	0.75
	2.7	1.48
	4.1	2.27
Reconstituted actomyosin	0.5	0.03
	1.0	0.08
	1.0	0.03
Natural acto-HMM S1	0.5	0.37
	1.0	0.78
	1.5	1.30
Reconstituted acto-HMM S1	0.3	0.09
	0.7	0.15
	1.0	0.09

Table 3

Comparison of the effect of myosin ATPase modifiers on the medium ^{18}O-exchange reaction catalyzed by myosin and HMM S1 in the absence of ATP

Preparation	Mg^{2+}-ATPase activity μmole P_i mg^{-1} × min^{-1}	Number of ^{18}O atoms per P_i molecule in the presence of	
		Mg^{2+}	Mn^{2+} + ADP
Original myosin	0.01	0.20	3.8
Reconstituted actomyosin	0.05	0.21	3.2
TNP-myosin	0.06	0.40	3.7
Congo-myosin	0.01	0.20	3.7
Original HMM S1	0.08	0.14	2.9
PABC-HMM S1	0.20	0.27	2.1

These data support the idea that some additional factors in natural complexes facilitate transformation of the intermediate exchange into medium exchange during myosin–actin interaction.

The data obtained make it possible to suggest the following hypothetical sequence of phosphorylation and ^{18}O-exchange reactions in myofibrils splitting ATP (Fig. 1).

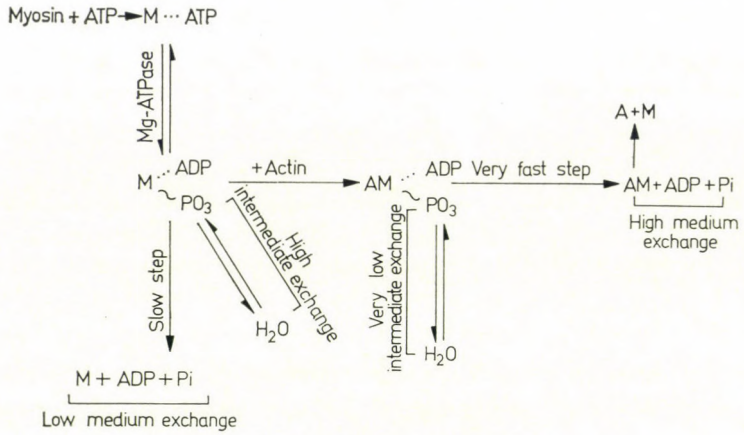

Fig. 1. Sequence of events during myofibrillar ATP splitting

Table 4

^{18}O-exchange reactions catalyzed by Na^+-K^+-ATPase from guinea pig kidney cortex in the course of ATP hydrolysis

Addition or treatment	ATPase activity μmole P_i $mg^{-1} \times min^{-1}$	Number of ^{18}O atoms per P_i molecule	
		Intermediate exchange	Medium exchange
None	0.56	0.74	0.92—2.8*
Deoxycholate (0.04%)	0.38	1.05	0
Aceton (20%)	0.36	1.34	0.11
Storing at 4° for 4 months	—	1.94	0.76
Urea (1.3 M)	0.46	2.94	0.27

* The values were observed in the absence of ATP, and they are proportional to protein concentration (0.1—0.4 mg × ml^{-1})

In the relaxed muscle, when myosin and actin filaments are separated, very slow reaction of ATP hydrolysis takes place under the ionic conditions prevailing in muscle. In this state myosin accumulates the energy by intermediate phosphorylation. Since the life-time of the phosphorylated protein is sufficiently long, the intermediate ^{18}O-exchange reaction is rather intense. It has enough time to proceed. Medium exchange with myosin as such is negligible.

Upon stimulation and under conditions that favour the combination of actin and myosin filaments, the rate of ATP splitting is significantly enhanced. The life-time of phosphorylated myosin markedly decreases. In this state the inter-

mediate exchange is very small because of the time limits. Simultaneously, medium oxygen exchange is stimulated irrespective of whether ATP is present or not.

When cross-bridges are broken by a new molecule of ATP which binds to myosin, a new cycle of ATP splitting and energy accumulation by myosin can begin at the expense of slow phosphorylation.

Both types of ^{18}O-exchange reactions which were observed with myosin and actomyosin are catalyzed by Na^+-K^+-dependent ATPase preparations from guinea pig kidney cortex. It seems interesting to note that with fresh preparations of ATPase only high medium ^{18}O-exchange is observed up to 2.8 atoms ^{18}O per P_i molecule (Table 4). Intermediate exchange is small. Aging of preparation or the addition of substances disturbing lipoprotein and protein–protein interactions (deoxycholate, acetone, urea) will induce an increase in intermediate exchange to 2.0 – 3.0 atoms per P_i molecule (Table 4). So, there are some similarities in the mechanism of hydrolysis of ATP by actomyosin (myofibrillar) and Na^+-K^+-dependent (membrane) ATPase.

A similar role of the phospholipid components was suggested by Tonomura (1972) in the mechanism of phosphate dissociation step of the sarcoplasmic reticulum ATPase.

The discovery of ^{18}O-exchange reactions with membrane ATPase preparations (Skvortsevich et al., 1972), for which the existence of phosphorylated intermediates is well established, supports the still somewhat controversial assumption of ^{18}O-exchange reactions being indicative of phosphorylated intermediates.

The type of ^{18}O-exchange reaction can be considered as a criterion for the identification of myosin and actomyosin-like proteins in motile systems.

References

Ashhmarin, I. P. (1951) Biochimia *16* 259
Bagshaw, C. R., Trentham, D. R. (1973) Biochem. J. *133* 323
Biró, N. A., Coelho, R., Ehrlich, E., Guillain, F., Dvonc, C. (1973) Europ. J. Biochem. *40* 527
Boyer, P. D. (1964) in: Biochemistry of Muscle Contraction. J. Gergely (ed.). Little, Brown & Co., Boston, p. 94
Boyer, P. D. (1967) in: Current Topics in Bioenergetics, Vol. 2. Acad. Press, New York, p. 99
Boyer, P. D., Graves, D. J., Suelter, C. H., Dempsey, M. E. (1961) Analyt. Chem. *33* 1907
Fábián, F., Mühlrad, A. (1968) Biochem. Biophys. Acta *162* 596
Fiske, C. H., SubbaRow, I. (1925) J. Biol. Chem. *66* 375
Ilin, L. A. (1966) Vestn. Leningrad Univ., Ser. Biol. *3* 85
Ilin, L. A., Panteleeva, N. S. (1967) Tzitologia, *9* 553
Ivanov, I. I., Yuryev, V. A. (1961) Biokhimia i Patobiokhimiya Myshts, Medgiz, Moscow—Leningrad.
Itzhaki, D. F., Gill, H. M. (1964) Analyt. Biochem. *9* 401
Koshland, D. E., Levy, H. M. (1964) in: Biochemistry of Muscle Contraction. J. Gergely (ed.). Little, Brown, & Co., Boston p. 87
Levy, H. M., Koshland, D. E. (1959) J. Biol. Chem. *234* 1102
Levy, H. M., Ryan, E. R., Springhorne, L., Koshland, D. E. (1962) J. Biol. Chem. *237* PC 1730

Liu-Osheroff, P., Guillory, R. J. (1972) Biochem. J. *127* 419
Lowey, S., Slayter, H., Weeds, A., Baker, H. (1969) J. Mol. Biol. *42* 1
Panteleeva, N. S. (1969) Second All-Union Biochem. Confer., Tashkent, 1969. Abstracts of Reports of Symposium, Publ. FAN, Uzbekistan SSR p. 253
Panteleeva, N. S., Karandashov, E. A., Kuleva, N. V. (1972) V. Internat. Biophys. Congress, Moscow, 1972. Section Reports, v. 4.
Pisareva, L. N. (1968) Tzitologia *10* 988
Post, R. L., Sen, A. K. (1967) in: Methods in Enzymology. Eastbook, R. W. and Pullman, M. E. (eds). Vol. X. Acad. Press, New York, p. 762
Skvortsevich, E. G., Panteleeva, N. S., Pisareva, L. N. (1972) Dokt. AN SSSR *206* 240
Swanson, J. R., Yount, R. C. (1966) Biochem. Z. *345* 395
Szent-Györgyi, A. G. (1953) Arch. Biophys. Biochem. *42* 305
Tonomura Y. (1972) Muscle Proteins, Muscle Contraction and Cation Transport. University Tokyo Press.
Yount, R. C., Koshland, D. E. (1963) J. Biol. Chem. *238* 1708

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Terminal Amino Acids, Peptide Map and Amino Acid Composition of One Form of Tomato Pectinesterase

(Short Communication)

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Pectinesterase (EC 3.1.1.11) – pectin-pectyl hydrolase, an enzyme catalysing the demethylation of pectin – is present in all higher plants tested so far (Kertesz, McColloch, 1950; Duel, Stutz, 1958). Pectinesterase present in multiple form in tomatoes was earlier described (Markovič, Slezárik, 1969; Pressey, Avants, 1972) and the major component of the five forms detected by starch-gel electrophoresis was isolated and partially characterized (Markovič, 1974).

In the present paper the terminal amino acids, amino acid composition and peptide map of the tryptic digest of the carboxymethylated enzyme isolated from *var. Immuna* is described.

N-terminal analysis

The N-terminal amino acid was determined by the dinitrophenyl method of Sanger (1945).

Direct identification of the DNP-amino acid was performed by chromatography on chromatosheet, precoated with silica gel (MN-Polygram, SIL-NHR, Macherey & Nagel Co., Düren, GFR) using the following solvent systems: chloroform–tert. amylalcohol–acetic acid (70 : 30 : 3) and benzene–pyridine–acetic acid (80 : 20 : 2) as described by Brenner et al. (1961). In these solvent systems we identified DNP-Ile, as the N-terminal amino acid.

Indirect identification was carried out after ammonolysis (Lowther, 1951) of the DNP-amino acid following its elution from the silica gel plate. The free amino acid was identified as Ile by thin-layer ion-exchange chromatography, on resin-coated chromatosheet (FIXION 50 × 8)* (Chinoin, Budapest, Hungary) using the method of Dévényi et al. (1971).

* The same results were obtained with IONEX-25 SA-NA (Macherey & Nagel Co., Düren, GFR).

Table 1

Amino acid composition of tomato pectinesterase[a]

Amino acid	Contents %	Molar ratio (M.W = 27500)	Nearest integer
Lysine	6.02	12.81	13
Histidine	1.02	2.02	2
Arginine	6.27	10.96	11
Aspartic acid	10.92	25.95	26
Threonine	6.61	17.82	18
Serine	4.66	14.72	15
Glutamic acid	10.02	21.19	21
Proline	4.20	11.81	12
Glycine	4.99	23.85	24
Alanine	6.19	23.79	24
Half-cystine[b]	1.50	3.97	4
Valine	6.13	16.90	17
Methionine	2.40	5.00	5
Isoleucine	4.53	10.91	11
Leucine	5.82	14.03	14
Tyrosine	7.23	12.08	12
Phenylalanine	4.90	9.08	9
Tryptophan[c]	2.73	4.02	4
Ammonid	3.84		

[a] Major component detected by starch-gel electrophoresis
[b] After oxidation with performic acid
[c] Determination by hydrolysis with thiolglycolic acid (Matsubara et al., 1969)

C-terminal analysis

The C-terminal amino acid of pectinesterase was determined by carboxypeptidase digestion, according to the micro-method of Sajgó and Dévényi (1972). About 0.1 μmol (2 – 3 mg) of the pectinesterase was dissolved in 0.1 M ammonium bicarbonate and digested by a mixture of carboxypeptidase A and B (Calbiochem) at 37 °C. After 0, 10, 20, 60 and 180 minutes samples were applied to resin-coated chromatosheet and developed in citrate buffer pH 3.3. The C-terminal amino acid was Leu, and the penultimate was Val.

Amino acid composition and peptide mapping

The amino acid composition was determined on automatic amino acid analyzer (Type 6020, A, Workshop of the Czechoslovak Academy of Sciences, Prague) by the method of Spackman et al. (1958). The results are summarized in Table 1.

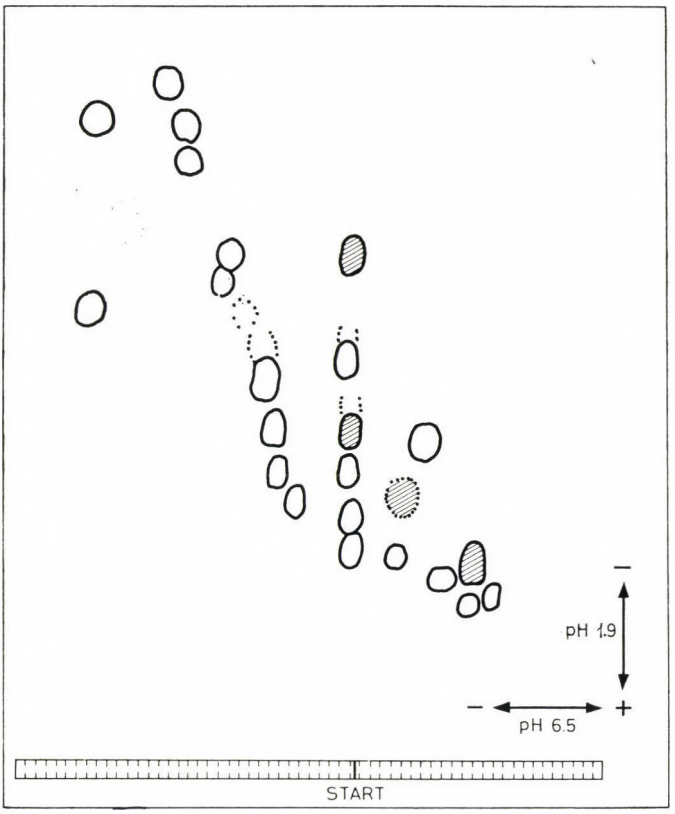

Fig. 1. Two-dimensional peptide map of the tryptic digest of fully carboxymethylated pectinesterase. Electrophoresis was performed in pyridine–acetic acid–water 100 : 4 : 896 (pH 6.5) for 1.5 hours at 50 V/cm, and in formic acid–acetic acid–water 20 : 80 : 900 (pH 1.9) for 2 hours at 80 V/cm. Black spots show the radioactive components revealed by autoradiography

The composition of tomato pectinesterase, isolated from *var. Hikari* was determined by Nakagawa et al. (1970). According to their analysis, cysteine, methionine and histidine are not present in the enzyme. The molar ratios presented here also differ slightly from our own data published earlier (Markovič, Slezárik, 1969), the reason of this discrepancy being due mainly to the revision of the molecular weight value (Markovič 1974).

The peptide mapping of the tryptic digest of a protein can provide some information as to the molecular weight by comparing the lysine and arginine content with the number of peptides revealed by electrophoresis and chromatography. Similar conclusion can be drawn from the number of radioactive peptide components revealed by autoradiography if the number of half-cystine in the molecule is known and the protein is fully carboxymethylated with [^{14}C]-bromoacetate before tryptic digestion.

Pectinesterase was carboxymethylated in the presence of 8 M urea with [^{14}C]-bromoacetate according to the method of Crestfield et al. (1963). After dialysis against 0.001 M HCl the carboxymethylated protein was digested with trypsin (Calbiochem, 1/50 by weight) at pH 8.2 for 2 hours, in 0.1 M ammonium bicarbonate. The freeze-dried digest was applied to Whatman 3 MM paper and separated by two-dimensional electrophoresis, using pyridine-acetic acid-water 100 : 4 : 896 (pH 6.5) and formic acid–acetic acid–water 20 : 80 : 900 (pH 1.9) buffers, at 50 V/cm and 80 V/cm, respectively.

After staining with ninhydrin, 24 peptide spots were detected (Fig. 1).

The paper chromatography of the neutral peptide band did not give further resolution.

The autoradiogram of the fingerprint showed the presence of four radioactive spots. The number of radioactive peptides in the tryptic digest, as well as the number of peptide spots are in good agreement with the number of half-cystine (4), arginine + lysine (11 + 13) residues, calculated for a molecular weight of 27 600 Daltons. This molecular weight value also agrees with the data obtained by Dellincée and Radola (1970) and with our previous finding (Markovič, 1974).

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References

Brenner, M., Niederwasser, A., Pataki, G. (1961) Experientia *17* 145
Crestfield, A. M., Moore, S., Stein, W. H. (1963) J. Biol. Chem. *238* 622
Dellincée, H., Radola, B. J. (1970) Biochim. Biophys. Acta *214* 178
Duel, H., Stutz, E. (1958) Adv. Enzymol. *20* 341
Dévényi, T., Hazai, I., Ferenczi, S., Báti, J. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. *6* 385
Kertesz, Z. I., McColloch, R. J. (1950) Advan. Carbohyd. Chem. *5* 79
Lowther, A. G. (1951) Nature *167* 767
Markovič, O., Slezárik, A. (1969) Collect. Czech. Chem. Commun. *34* 3820
Markovič, O. (1974) Collect. Czech. Chem. Commun. *39* 908
Matsubara, H., Sasaki, R. M. (1969) Biochem. Biophys. Res. Commun. *35* 175
Nakagawa, H., Yanagawa, Y., Takehana, H. (1970) Agr. Biol. Chem. *34* 998
Pressey, R., Avants, J. K. (1972) Phytochemistry *11* 3139
Sajgó, M., Dévényi, T. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. *7* 233
Sanger, F. (1945) Biochem. J. *39* 507
Spackmann, D., Stein, W. H., Moore, S. (1958) Anal. Chem. *30* 1190

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Study on the Biological Effect of Fast Neutrons

1. Effect of Fast Neutrons on Germinability of Barley Seeds and on the Chlorophyll Content of the Seedlings According to the Dose Used

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Barley seeds were irradiated in the active zone of the reactor at 500 watt, at favourable flux of fast neutrons in the dose interval of 823–2144 rad. Seeds after irradiation were "cooled" for 48 hours, then germinated, and the length of shoots of the seven days old seedlings was measured. The value of the half lethal dose is 1130 rad, estimated from the growth curve. There is a well-defined relation of chlorophyll content (calculated for fresh weight unit) to the dose used. It was concluded from the phenotype of the leaves that the decrease of chlorophyll content was due first of all to the presence of chlorophyll-less cells and cell lines and not to the decrease of chlorophyll content of the individual cells. Irradiation yielded genetically defected cells.

Introduction

Irradiations have a multifold effect on living systems. There is practically not even one feature or quality which remains constant in living organisms after exposure to irradiation. Studies on the biological effects of irradiations are, therefore, justified.

Comparison of the biological effects of different kinds of irradiations shows neutrons to cause deeper changes in living organisms than X- or gamma-rays. The considerable biological effect of neutrons is interpretable from their corpuscularity and other physical characteristics.

Studies on the effect of fast neutrons are followed with distinguished interest. Atomic energy power stations and experimental atomic reactors which are to be built in increasing number, and the frequent space flights further justify the studies of effects of fast neutrons on living organisms.

According to our knowledge, there are relatively few papers on studies of the effects of fast neutrons on higher plants. From the Technical Reports of the International Atomic Energy Agency it is clear that the aim of the first experiments made with higher plants (chiefly barley and wheat) has been to characterize the

dose of irradiation of the various types of reactors (Luse, Brunner, 1972). The basis of comparison of these results is the decrease of germinability of seeds and the inhibition of growth of seedlings, respectively. This type of experimental work made it possible to select the proper radiation types and doses which result in easily characterizable biological effects.

It was shown by Bjornseth et al. (1957) that the retardation of growth caused by exposure to irradiation was connected with the inhibition of respiration and protein synthesis. Several other physiological and biochemical effects were also observed (Mikaelsen et al., 1956; Celishchev, Mogilevskii, 1957; Valeva, 1960; Golikova, 1969; Koo et al., 1972).

Many workers point out the genetical effect of fast neutrons (Domini et al., 1972; Gopal-Ayengar et al., 1972; Savage et al., 1972; Stoilov et al., 1972). Generally two different test methods are used for examination of genetical changes: 1) cytological analysis (e.g. examination of chromosomes) for detection of direct genetical damages, or 2) formation of more productive species or varieties for breeding purposes.

The present paper deals with the study of the biological effects of neutrons, as a function of dose, performed on barley seeds. The effect of different doses of fast neutrons on the growth and chlorophyll content of barley seedlings was studied.

Materials and methods

Air-dry barley seeds (Tápláni, spring var.) were used in the experiments. The seeds were irradiated in the active core of the reactor. Fig. 1 shows a sketchy map of the reactor core in the height of the axis line of the horizontal channels. The core was built of fuel elements of type EK-10 containing ^{235}U isotope in 10 per cent enrichment (made in USSR). The quantity of ^{235}U in the core was 2824 g.

Seeds were irradiated in the channel operated through a pneumatic rabbit facility (marked "h" in Fig. 1).

At the place of irradiation, the ratio of fast to thermal neutron flux is very advantageous for reasons of reactor physics (Fig. 2). The sample which consisted of 50 barley seeds were held in a plastic container (inside diameter 11 mm, length 45 mm) and this container was put into the pneumatic rabbit facility.

To characterize the dose situation, concepts of absorbed and kerma dose are used in neutron dosimetry. There is no place here to go into the discussion of the physical differences of these two dose quantities, but it should be remarked that in case of irradiation of small-size samples (as e.g. barley seeds) the numerical values of the two doses can be considered equal within the limits of the experimental errors.

The following steps are used for the determination of neutron doses: 1. the fluence, determined on the basis of the flux values, known to be a function of the power of the reactor, and of the irradiation time; 2. the relation between the

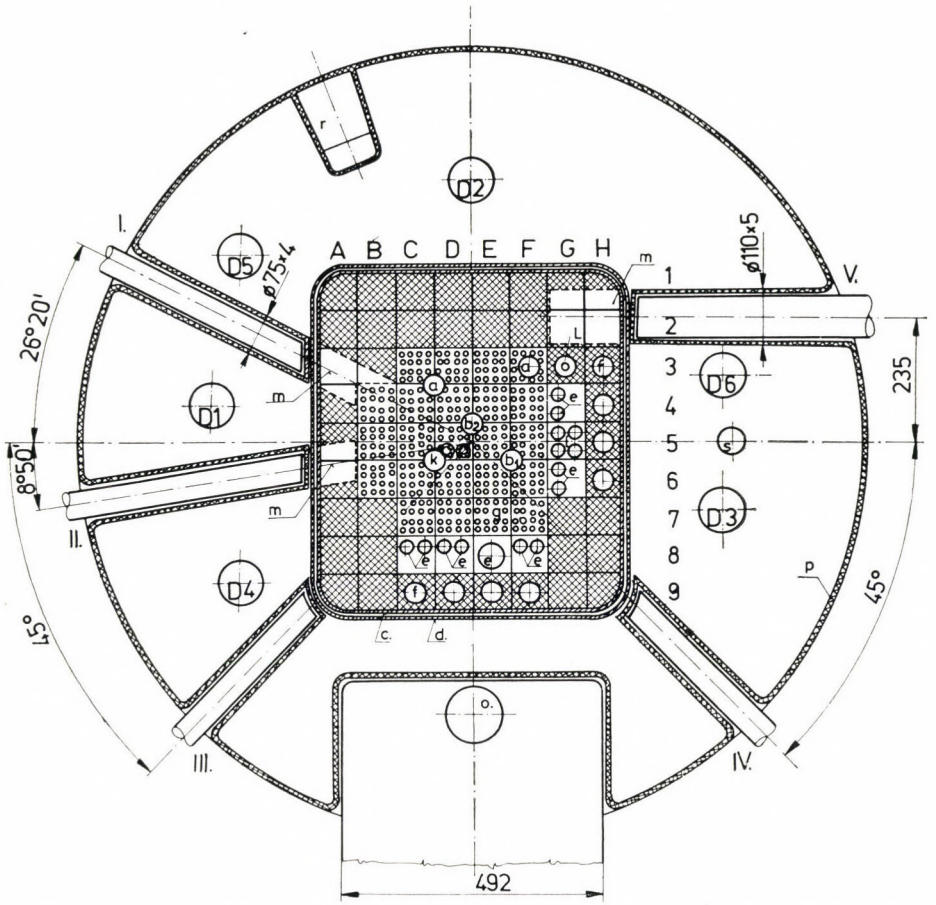

Fig. 1. Cross section of the container of the reactor in the height of the axis line of the horizontal tubes

fluence and the kerma, given by Eq. (1):

$$K = \bar{d}_k \cdot \Phi \tag{1}$$

where K = kerma dose (rad)
Φ = fluence ($n \cdot cm^{-2}$)
$\bar{d}_k$ = fluence–kerma conversion factor ($rad \cdot cm^2$).

For a spectrum of neutrons (which is the case for a reactor), $\bar{d}_k$ depends on the mean energy of the neutrons.

The values of $\bar{d}_k$ can be found in tables (Nachtigall, 1970). The kerma dose also depends on the chemical composition of the sample.

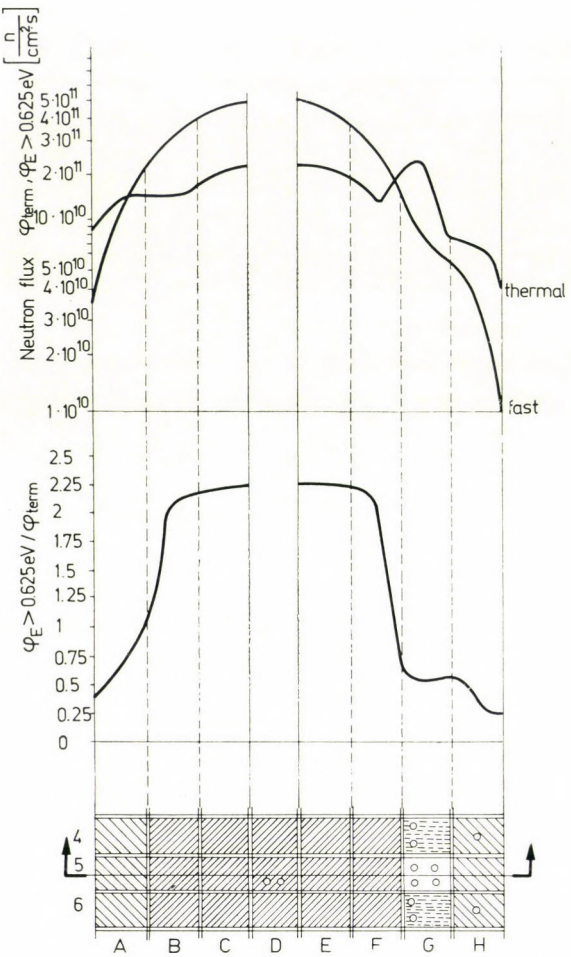

Fig. 2. Ratio of fast neutron flux to thermal neutron flux in the active zone of the reactor

Supposing that the mean energy of the neutrons does not essentially deviate from the mean energy of the fission spectrum and that the irradiated biological material mainly contains elements of small atomic number, we may take Eq. (2):

$$\bar{d}_k = 2.77 \cdot 10^{-9} \text{ rad} \cdot \text{cm}^2 \tag{2}$$

The irradiations were carried out at a power of 500 watt. According to the data available, the flux of neutrons with $\bar{E} \cong 1$ MeV energy was $\varphi = 1.36 \cdot 10^{10}$ n · $\text{cm}^{-2}\text{sec}^{-1}$, and:

$$\Phi = \varphi \cdot t = 1.36 \cdot 10^{10} \text{ n} \cdot \text{cm}^{-2} \text{ sec}^{-1} \quad t \text{ (sec)} \tag{3}$$

Table 1

Correlation between duration of irradiation, integral flux and kerma dose in experiments on barley seeds exposed to fast neutrons

Duration of irradiation (sec)	Integral flux $a = \phi \cdot t$ $\left(\frac{n}{cm^2}\right) \cdot 10^{11}$	Kerma dose $K = \Phi \cdot \bar{d}_k$ (rad)
22	2.99	823
29	3.94	1090
36	4.88	1350
43	5.86	1458
50	6.81	1622
57	7.74	2144

From the above values of $\bar{d}_k$ [Eq. (2)] and Φ [Eq. (3)] the calculated kerma dose values are tabulated in Table 1 for irradiation times t = 22, 29, 36, 43, 50 and 57 sec.

The irradiated barley seeds were cooled for 24 hours then were germinated on moisted paper wedding in an illuminated incubator at 25°C. The seven days old seedlings were treated as follows:

1. length of shoot was measured for the determination of the extent of growth;

2. the chlorophyll content was estimated on fresh weight basis.

For estimation of chlorophyll content a spectrophotometric method was used with a two-wavelength technique. The quantity of chlorophyll-*a* and chlorophyll-*b* was calculated from the optical density of ether extracts by the use of the following empirical connections (French, 1960):

chlorophyll-*a* = $11.44 \cdot D_{663} - 0.96 \cdot D_{644}$ nmol/ml·cm;

chlorophyll-*b* = $18.01 \cdot D_{644} - 2.90 \cdot D_{663}$ nmol/ml·cm.

All the results given are statistically processed data of 3 independent series of estimations.

Results and discussion

Fig. 3 shows the inhibitory effect of fast neutrons on the growth of barley seedlings. It can be seen that there is a well-defined correlation between dose and extent of growth. This correlation does not continue over the dose of 1622 rad, the curve becomes flat and, at a dose of about 2144 rad, the growth stops finally. The $LD_{50/30}$ calculated from the dose-effect curve is about 1130 rad, which corresponds to an irradiation of 30 sec duration.

The half lethal dose was found to be ca. 1100–1300 rad in similar experiments but with other types of reactors according to the literature (Gopal-Ayengar et al., 1972; Stoilov et al., 1972). Our experimental data are in good agreement with other data published.

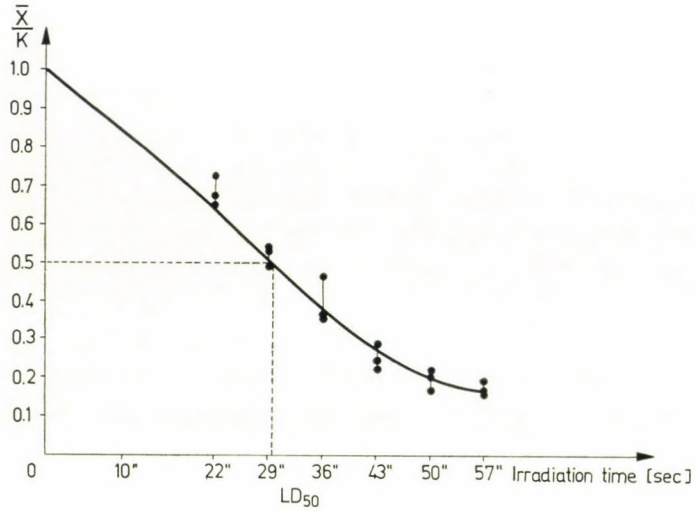

Fig. 3. The change of shoot length of barley seedlings as a function of the duration of irradiation. (In per cent of the control plants)

With respect to the circumstances of the exposure, it should be mentioned that the irradiation was performed two days after the reactor had been started up. This means that the level of accompanying gamma radiation was very low (about 1000 R), i.e. by several orders of magnitude lower than the half lethal dose of gamma radiation known from the literature to be about 60 000 rad.

Presumably, our variety of barley seeds was more sensitive to irradiation than the varieties used in other laboratories. Gopal-Ayengar et al. (1972) have found different sensitivities in various plant species and varieties against irradiation with fast neutrons. Their data show a wide diversity of the biological effect of fast neutrons in the different objects used in their experiments. The half lethal doses given by them were in the same order of magnitude as are our LD_{50} values.

Table 2

The change of total chlorophyll content in dependence on the doses used

Duration of irradiation (sec)	Chlorophyll $a + b$ percentage	Chl-*a*/Chl-*b*
0	100.0	3.0
22	97.6	3.0
29	93.6	2.9
36	81.0	2.9
43	59.9	2.9
50	50.2	2.9
57	43.1	3.4

Table 2 shows the chlorophyll content of the seedlings depending on the dose, calculated on fresh weight basis.

As it can be seen from Table 2 the percentage chlorophyll contents are decreased when compared with the control plants, in dependence of the dose used. The decrease of the chlorophyll content is over 50 per cent at the highest radiation dose. Chlorophyll-*a* and chlorophyll-*b* are affected roughly to the same extent; this can be seen from the ratio of Chl-*a* : Chl-*b*.

The loss of chlorophyll content upon the effect of irradiation is well perceptible from the appearance of leaves. According to our results, the loss of chlorophyll in the cells is not uniform; completely chlorophyll-less cells and cell-lines turn up together with normal cells. There is a mosaic pattern on the surface of barley leaves caused by chlorophyll-containing and chlorophyll-deficient cell lines. From the mosaic pattern of the cell lines the conclusion can be drawn that the cells present in the leaves are genetically different. This is an essential result, because a well-definable genetical abnormity appears in the first (F_1) generation and there is no need to study further (F_2, F_3) generations.

Nicoloff and Georgieva (1975) have shown with chlorophyll-deficient barley mutants induced by ethyl methanesulphonate (EMS) that the frequency of leaves with chlorophyll-deficient sectors is equal to the frequency of the chlorophyll-deficient mutants in F_2.

References

Bjornseth, J., Goksoyr, J., Mikaelsen, K. (1957) Physiol. Plant. *10* 328

Celishchev, S. P., Mogilevskii, V. B. (1957) Izvestija Timirjazevskoj s-h. akademii *3* 33

Domini, B., Bozzini, A., Brunori, A., Avanzi, S., Scarescia-Mugnozza, C. T. (1972) Neutron irradiation of seeds. III. Technical reports series No. 141, IAEA, Vienna, p. 91

French, C. S. (1960) In: Handbuch der Pflanzenphysiologie, p. 252

Golikova, O. P. (1969) In: Biologischeskoe dejstvie bistrih neutronov *1* 115

Gopal-Ayengar, A. R., Rao, N. S., Bhatt, B. Y., Mistry, K. B., Joshua, D. C., Thakare, R. G. (1972) Neutron irradiation of seeds. III. Technical reports series No. 141. IAEA, Vienna, p. 1

Koo, R. K. S., Ferrer-Monge, J. A., Otero, A. M., Bulla, I., Alemany, A., (1972) Neutron irradiation of seeds III. Technical reports series No. 141, IAEA, Vienna p. 95

Luse, R. A., Brunner, H. (1972) Neutron irradiation of seeds III. Technical reports series No. 141. IAEA, Vienna, p. 45

Mikaelsen, K., Bjornseth, S., Halvosen, H. (1956) Physiol. Plant *9* 697

Nachtigall, D. (1970) Physikalische Grundlagen für Dosimetrie und Strahlungschutz. Thieme Verlag, München

Nicoloff, Hr., Georgieva, S. (1975) Genetika *11* 47

Savage, J. R. K., Wigglesworth, D. J., Pritchard, U. A. (1972) Neutron irradiation of seeds. III. Technical reports series No. 141, IAEA, Vienna, p. 87

Stoilov, M., Filev, K., Thoehev, T., Damianov, D., Georgiev, R. (1972) Neutron irradiation of seeds. III. Technical reports series No. 141. IAEA, Vienna, p. 21

Valeva, S. (1960) Biofizika *5* 362

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Transmission of Excitation from Muscle to Nerve under *in situ* Conditions*

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In order to investigate the excitatory effect of the muscle on the adjacent nerve experiments were performed on nerve-muscle and whole leg preparations of the frog. The results showed that muscular activity could generate excitation of the nerve in contact with the muscle under *in situ* circumstances, and that a well-defined part of the muscle could play a significant role in the transmission of excitation from muscle to nerve. The results support the hypothesis of being an additional way for muscle activity to influence the regulatory mechanism of the nerve-muscle function during excitatory processes.

Introduction

In spite of the great number of researches in physiology and biophysics back to the last century, concerning connections of excitable tissues, the significance of non-synaptic interactions between the excitable elements has not yet been clarified.

In his classical experiments Matteucci (1842) demonstrated that an active muscle could generate excitation in the nerve in contact with the muscle, and Hering (1882) showed that, in special circumstances, the activity of a nerve fibre could stimulate another fibre within the common nerve trunk.

Later, the stimulatory effect of an active nerve fibre on an inactive one was only demonstrated in the case of non-myelinated nerves (Jasper, Monnier, 1938; Arvanitaki, 1942), or in myelinated nerves with artificially increased excitability (Rosenblueth, 1941).

As to the transmission of excitation from muscle to nerve different nerve activities were recorded which showed that the muscle could exert an excitatory effect on the motor and sensory nerve fibres inside the muscle (Masland, Wigton,

* This paper which was prepared in the Institute directed by Academician J. Tigyi, member of the Editorial Board of this journal, and the publication of which has been recommended by him but was strongly opposed by the professional referee, a distinguished scientist, is considered by me an appropriate object for a public debate of experts. (*E. Ernst*).

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1940; Eccles et al., 1942; Lloyd, 1942; Leksell, 1945; Granit et al., 1959; Brown, Matthews, 1960; Werner, 1961; Epstein, Jackson, 1970).

The results obtained on isolated nerve-muscle preparations and on whole leg preparations of frog proved that, under certain conditions, the action potential of the muscle is capable of producing excitation of the nerve adjacent to the muscle (Biró, 1975).

In the present work our previous experiments have been continued in order to investigate the excitatory effect of muscle activity on the nerve contact with the muscle under *in situ* conditions.

Methods

The experiments were performed on frog's (*Rana esculenta*) nerve-muscle preparation containing the sciatic nerve, the m. gastrocnemius, the r. superficialis n. tibialis and the m. flexor superficialis. The r. superficialis n. tibialis is that branch of sciatic nerve that is situated on the m. gastrocnemius and innervates the m. flexor superficialis. The m. flexor superficialis was not isolated from the foot but the connections of the foot with the m. gastrocnemius were only the Achilles tendon and the r. superficialis n. tibialis. In one part of the experiments the whole leg together with the sciatic plexus was used.

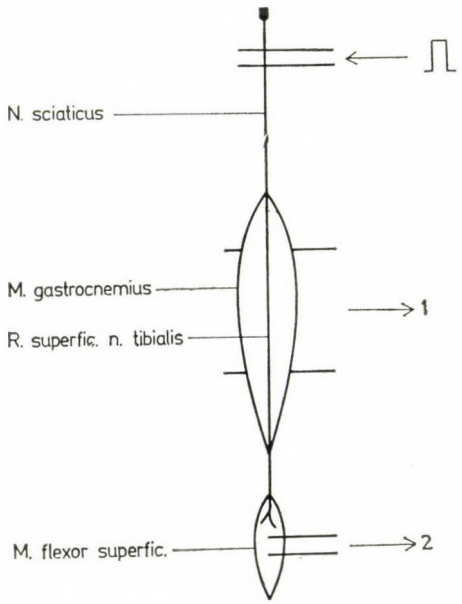

Fig. 1. Schematic diagram of the preparation and its connections with the electrodes

The responses of preparations were elicited by square-wave impulses of 0.1 ms given to the sciatic nerve or sciatic plexus. The electrical activity of the muscle was recorded by oscillographic method. Electrode pairs made from platinum wire 1 mm in diameter were used for stimulation and recording the action potential. The distances between the two electrodes on the nerve, the m. gastrocnemius and the m. flexor superficialis were 5, 20 and 2 mm, respectively. In some experiments the action potentials of the m. flexor superficialis were recorded with platinum electrodes 0.2 mm in diameter. The distance between these electrodes was 0.5 mm. The preparation and its connections with the electrodes are shown schematically in Fig. 1.

The action potential of m. gastrocnemius (1) and the m. flexor superficialis (2), respectively, were recorded by the upper and the middle beam of the oscilloscope. The only exception was one series of experiments (Fig. 9) where the middle beam also recorded the electrical activity of the m. gastrocnemius. The stimulatory impulses were also recorded on the lower beam as a point above the time markers.

The experiments were carried out at room temperature (20–24 °C). Care was taken to keep the preparations under wet condition.

Results

The excitatory effect of action potential of m. gastrocnemius on the nerve branch r. superficialis n. tibialis was investigated in different ways. One of the methods for demonstrating this excitatory effect was the application of ligature on the nerve affected by the electrical activity of the muscle. In this kind of experiments a comparison was made between the latencies of the action potentials recorded from the m. flexor superficialis before and after ligation of the r. superficialis n. tibialis. As shown in Fig. 2 the latency of the action potential of the m. flexor superficialis appears to be about 4 ms longer in the case of the ligated nerve than in the case of the intact preparation. The longer latency is due to the effect of ligature arresting the conduction of excitation produced by the square-wave stimulus given to the sciatic nerve. In spite of the conduction block, the r. superficialis n. tibialis had to become excited below the ligature because the m. flexor superficialis responded with a delayed action potential. The reason of this delayed response is that an excitation could be generated in the distal portion of the r. superficialis n. tibialis by the action potential of the m. gastrocnemius. In some experiments the conduction block was brought about by cutting the nerve instead of ligating it. The results obtained with cutting the r. superficialis n. tibialis were the same as those demonstrated in Fig. 2.

In the case of the ligated r. superficialis n. tibialis the delayed response of m. flexor superficialis was also demonstrated in such a way that stimulus was given to the m. gastrocnemius directly in spite of the stimulation of the sciatic nerve (Fig. 3). When the m. gastrocnemius was stimulated with 1 V only the delayed

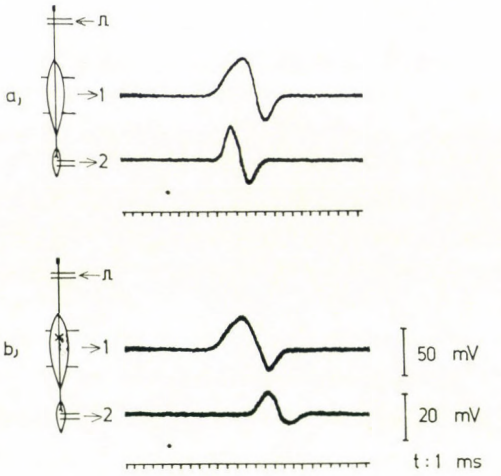

Fig. 2. Action potentials of m. gastrocnemius and m. flexor superficialis: a) before ligature; b) after ligature

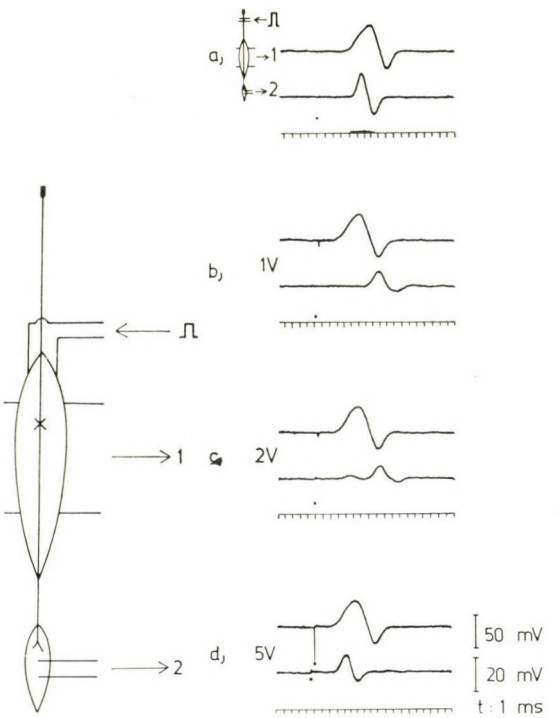

Fig. 3. Electrical activity of m. gastrocnemius and m. flexor superficialis: a) before ligature — indirect stimulus of 2 V; b) after ligature — direct stimulus of 1 V; c) after ligature — direct stimulus of 2 V; d) after ligature — direct stimulus of 5 V

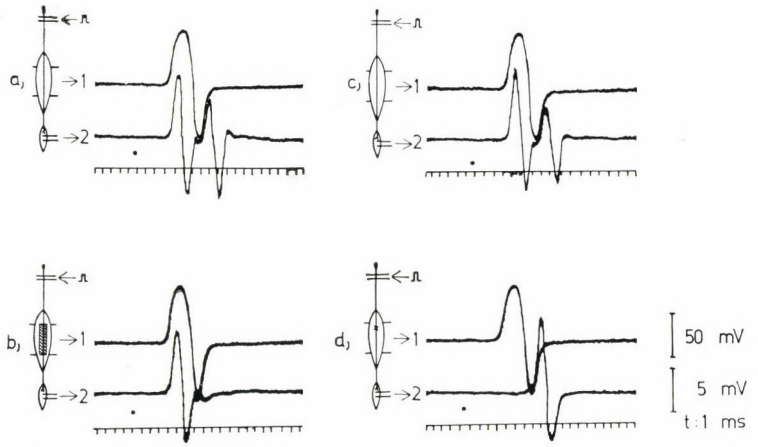

Fig. 4. Action potentials recorded from m. gastrocnemius and m. flexor superficialis: a) responses of intact preparation; b) responses obtained after insulating m. gastrocnemius from r. superficialis n. tibialis by means of a nylon foil; c) responses after removing nylon foil; d) responses after ligature

action potential of the m. flexor superficialis could be recorded (Fig. 3b). Increasing the stimulus amplitude to 2 V doubled the action potential of the m. flexor superficialis (Fig. 3c). To stimulation of the m. gastrocnemius with a square-impulse of 5 V in amplitude the response of m. flexor superficialis was only a single action potential (Fig. 3d). In the case of Fig. 3c, only a part of the fibres and in the case of Fig. 3d all of the fibres in the distal position of the r. superficialis n. tibialis could be directly stimulated with the electrical impulse. Comparison of the latencies of the responses of the m. flexor superficialis proves that the action potential shown in Figs 3a and 3d as well as the first action potential in Fig. 3c were produced with the nerve excitation generated by the electrical impulse; the action potential in Fig. 3b, and the second action potential in Fig. 3c could be produced with the nerve excitation generated by the action potential of the m. gastrocnemius.

The other method for demonstrating the stimulatory effect of muscle excitation on the neighbouring nerve was realized in those experiments in which a single stimulus given to the sciatic nerve of an intact preparation produced two action potentials (Fig. 4a). The separation of these two action potentials pointed to a difference in the mechanisms generating the first and the second response of the m. flexor superficialis. As it is shown in Fig. 4b, an about 0.1 mm thick nylon foil was placed between the m. gastrocnemius and the r. superficialis n. tibialis for electrical insulation of the nerve from the muscle tissue. In this case, only the first action potential could be recorded from the m. flexor superficialis. Removing the nylon foil doubled the response (Fig. 4c). It was also possible to record only the second action potential by means of application of the ligature mentioned above (Fig. 4d). From the results shown in Fig. 4 it is concluded that the second action

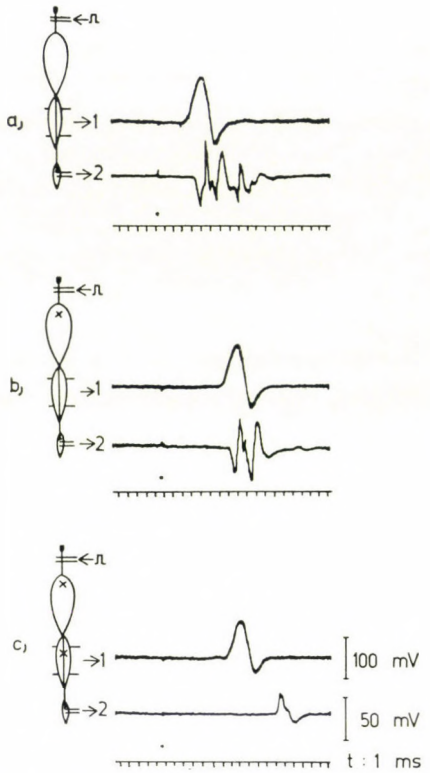

Fig. 5. Action potentials recorded from m. gastrocnemius and m. flexor superficialis in the case of whole leg preparation: a) before ligatures; b) after ligature of the sciatic nerve; c) after ligature of the sciatic nerve and the r. superficialis n. tibialis

potential of the m. flexor superficialis was a consequence of the excitatory effect exerted by the m. gastrocnemius on the adjacent nerve.

In the experiments demonstrated in Fig. 4, the action potentials of the m. flexor superficialis were recorded by means of the electrode pair 1 mm in diameter. The distance between the electrodes was 2 mm. Under such conditions the double response of the m. flexor superficialis could be recorded in 15 out of 25 experiments. In the next series of experiments other electroces were used for recording the action potentials of the m. flexor superficialis. These electrodes were made from platinum wire 0.2 mm in diameter and the distance between them was 0.5 mm. The application of these fine electrodes enabled us to find such a place on the m. flexor superficialis from which the double response could be recorded regularly. The oscillogram obtained after ligation of the r. superficialis n. tibialis only showed the second action potential also in these cases.

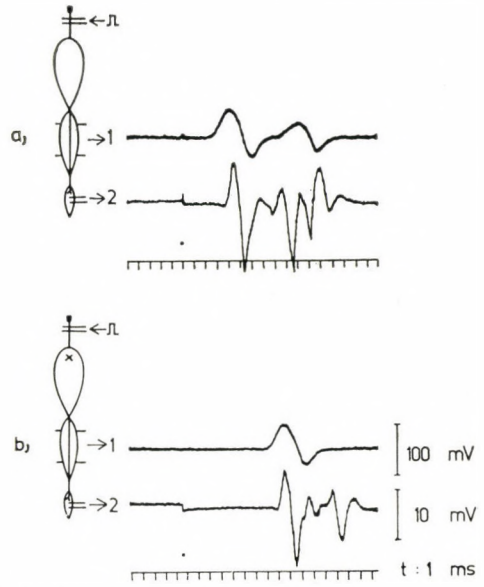

Fig. 6. Action potentials recorded from m. gastrocnemius and m. flexor superficialis in the case of whole leg preparation; a) before ligature; b) after ligature of the sciatic nerve

Experiments were also performed on whole leg preparations using the fine electrodes mentioned above. In these experiments a single square-wave stimulus given to the sciatic plexus generally produced three successive action potentials recorded from the m. flexor superficialis (Fig. 5a). Ligation of the sciatic nerve at the upper third of the thigh resulted in two changes (Fig. 5b). One of the consequences of this ligation was a longer latency of the action potentials in both records. The increase in the latency was about 3.5 ms. The other consequence was a decrease in the number of the action potentials recorded from the m. flexor superficialis. The application of an additional ligature on the r. superficialis n. tibialis brought about a further increase in the latency and another decrease in the number of the action potentials of the m. flexor superficialis. Occasionally, action potentials were also recorded from the thigh muscles. The latency of these action potentials was about 3 ms at all three stages of the experiments shown in Fig. 5. The results of Fig. 5 can be explained by the excitatory effect of the muscle activity on a neighbouring nerve. In the case of the intact leg preparation the triple response of the m. flexor superficialis could be produced by three successive stimuli to the nerve innervating this muscle. The first of the stimuli was the square-impulse, the second one the electrical activity of the femoral muscles and the third one the action potential of the m. gastrocnemius. These three stimuli exerted their effects, in order of time, on the sciatic plexus, on the part of the sciatic nerve among the muscles of the thigh and on the r. superficialis n. tibialis. The effects of ligations on the sciatic nerve and on the r. superficialis n. tibialis, respectively, manifested them-

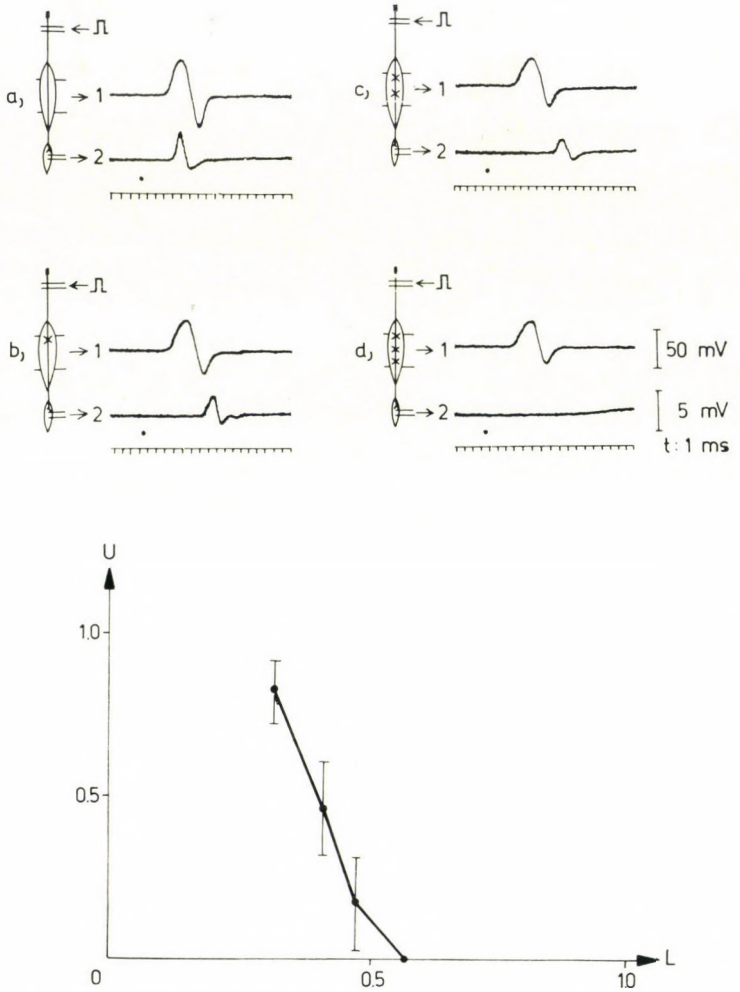

Fig. 7. Action potentials recorded from m. gastrocnemius and m. flexor superficialis: a) before ligature; b) after the first ligature; c) after the second ligature; d) after the third ligature. The diagram shows the dependence of the amplitude of the action potential of m. flexor superficialis on the length of r. superficialis n. tibialis below the ligature; U) mean and standard deviation of the peak-to-peak amplitude in relative unit; value 1 means the maximal amplitude; L) distance of the ligature from proximal end of m. gastrocnemius in relative unit; value 1 means the resting length of the muscle

selves in vanishing of the first and the second action potential recorded from the m. flexor superficialis. The increase in the latencies shown on the oscillograms was also produced by the block of conduction due to the ligatures.

In connection with the previous results it could be expected that double response will be recorded from the m. gastrocnemius because of the excitatory effect

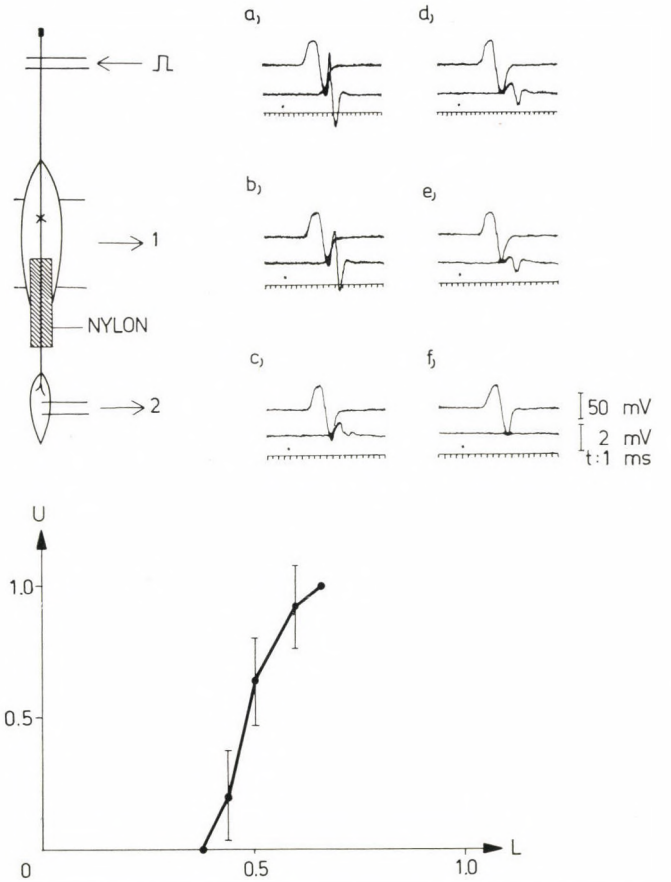

Fig. 8. Action potentials recorded from m. gastrocnemius and m. flexor superficialis after ligature. The records (a—f) were taken at different stages of approaching of the nylon foil the ligated place from the distal end of m. gastrocnemius. The diagram shows the dependence of the amplitude of the action potential of m. flexor superficialis on the length of r. superficialis n. tibialis between the ligature and the proximal edge of the nylon foil. U) mean and standard deviation of the peak-to-peak amplitude in relative unit; value 1 means the maximal amplitude; L) distance of the proximal edge of the nylon foil from the proximal end of m. gastrocnemius in relative unit; value 1 means the resting length of the muscle

of the thigh muscles on the sciatic nerve. In some experiments performed with intact leg preparation two action potentials were actually recorded from the m. gastrocnemius (Fig. 6a). After the ligation of the sciatic nerve at the upper third of the thigh the records from the m. gastrocnemius showed only one delayed action potential (Fig. 6b). The responses of the m. flexor superficialis were similar to those shown in Fig. 5.

In the following experiments changes were made in the contact of the m.

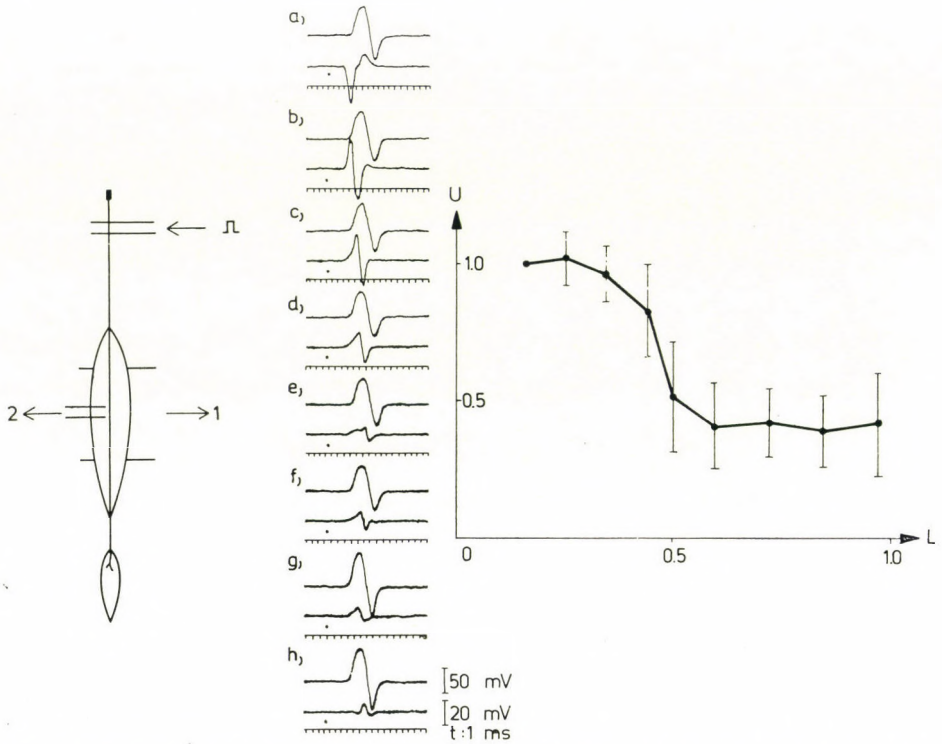

Fig. 9. Action potentials recorded from m. gastrocnemius by means of electrodes 1 (upper beam) and electrodes 2 (middle beam). The records (a—h) were taken in different positions of electrodes 2 moving in proximo-distal direction along r. superficialis n. tibialis. The diagram shows the change in amplitude of action potential recorded by electrodes 2. U) mean and standard deviation of the peak-to-peak amplitude in relative unit; value 1 means the maximal amplitude; L) distance of electrodes 2 from the proximal end of m. gastrocnemius in relative unit; value 1 means the resting length of the muscle

gastrocnemius with the r. superficialis n. tibialis in order to investigate the conditions modifying the excitatory effect of the muscle on the adjacent nerve.

In the first part of this series of experiment the length of the nerve capable of propagating the nerve excitation generated by the muscle activity was altered by means of several ligatures arranged successively at a few mm from one another. According to the oscillograms and the diagram in Fig. 7, decrease of the intact length of the r. superficialis n. tibialis caused a considerable reduction in the amplitude of the action potential recorded from the m. flexor superficialis. The last ligature was situated at the border line between the middle and lower third of the m. gastrocnemius. In spite of the fact that the nerve was in normal contact with the muscle at its lower third, there was no sign of the excitatory effect of the muscle.

The other way of changing the surface of contact of the nerve with the muscle was also accomplished by means of a 0.1 mm thick nylon foil. After ligature of

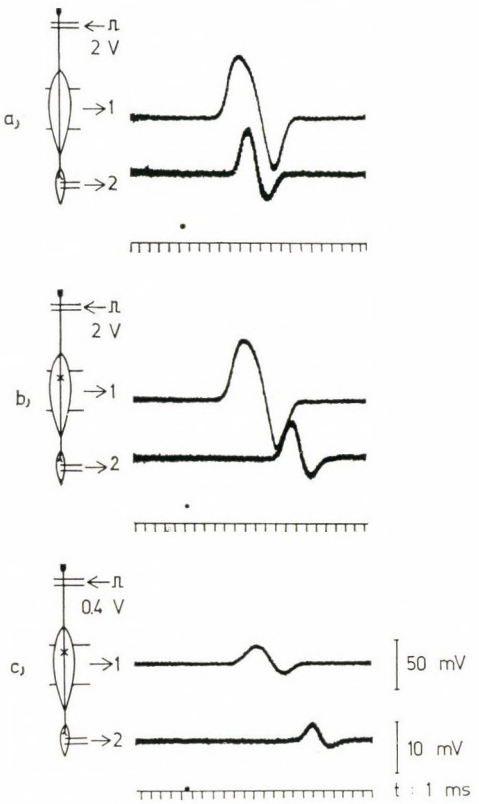

Fig. 10. Action potentials recorded from m. gastrocnemius and m. flexor superficialis: a) before ligature — stimulus of 2 V; b) after ligature — stimulus of 2 V; c) after ligature — stimulus of 0.4 V

the r. superficialis n. tibialis a nylon foil was placed under the lower part of this nerve and moved successively in proximal direction from the distal end of the m. gastrocnemius (Fig. 8). When the nylon foil insulated the nerve from the muscle only on its distal third, the amplitude of the action potential recorded from the m. flexor superficialis did not change. When the nylon foil approached the place of the ligature the amplitude showed a significant decrease in the course of shifting the nylon into the middle third of the m. gastrocnemius.

Investigating the reason why the middle part of the m. gastrocnemius plays a significant role in transmitting the excitatory effect on the nerve we recorded action potentials from different areas of the m. gastrocnemius. These areas were situated along the r. superficialis n. tibialis. As it can be seen in Fig. 9 two action potentials were recorded from the m. gastrocnemius. The first one was the usual record, the second one was registered by means of the electrodes 2 mm apart. The oscillograms and the diagram of Fig. 9 demonstrate that the amplitude of the

second action potential considerably decreased in the middle third and this low value remained nearly unaltered at the distal third of the m. gastrocnemius.

The results mentioned above point to the significance of the magnitude of the electrical potential produced by the muscle activity. In the previous experiments the activity was generated in the m. gastrocnemius by a supramaximal stimulus given to the sciatic nerve. Since supramaximal stimulation is not physiological, experiments were also performed with stimulation of lower intensities. The diminution in the intensity of the stimulus brought about a decrease in the amplitude of the action potential recorded from the m. gastrocnemius. The smaller activity of the m. gastrocnemius exerted a less pronounced excitatory effect on the part of the r. superficialis n. tibialis below the ligature. In this kind of experiments oscillograms were taken with supramaximal stimulus intensity and with that producing minimal action potential of the m. flexor superficialis (Fig. 10). Statistical analysis of the results obtained in 35 experiments showed that the minimal response of the m. flexor superficialis was generated at muscle activity corresponding to 24 ± 6 per cent of the amplitude of the action potential recorded from the m. gastrocnemius. Relying upon these findings, it can be concluded that activity of the m. gastrocnemius much smaller than the supramaximal one is sufficient for producing an excitatory effect on the r. superficialis n. tibialis.

Discussion

The present series of experiment showed that the muscle could have the ability to elicit excitation of the adjoining nerve under *in situ* circumstances.

The excitatory effect of the m. gastrocnemius manifested itself in two types of experiment. On the one hand, the m. flexor superficialis produced action potential in spite of the fact that the r. superficialis n. tibialis was ligated and the nerve excitation elicited by square-wave simulation of the sciatic nerve could not propagate through the ligated place (Biró, 1975). In this type of experiments the response of the m. flexor superficialis must have been a consequence of the excitatory effect of the m. gastrocnemius on the part of the r. superficialis n. tibialis below the ligature (Fig. 2). On the other hand, the double and triple responses of the m. flexor superficialis proved that the primary excitation of the r. superficialis n. tibialis could be followed by excitations generated by the activities of the muscles in contact with the nerve (Fig. 4 and Fig. 5).

The analysis of the results of Fig. 4 showed that the appearance of the double response could depend upon different factors, i.e. the threshold, the conduction velocity, the refractory period of the nerve and the position of the recording electrodes on the muscle. To clear the role of these factors further experiments are required.

The connection between the nerve and muscle is an important factor because the transmission of excitation from muscle to nerve does not take place

in every position of the nerve on the muscle (Biró, 1975). The experimental results demonstrated in Figs 7–9 show that the middle third of the m. gastrocnemius is able to excite the r. superficialis n. tibialis, while with the distal third this is not the case.

It was demonstrated in experiments performed on isolated nerve-muscle preparations that the submaximal activity of the muscles could represent an effective stimulus for the nerve laying on the muscle (Biró, 1975). This finding is corroborated by the results of Fig. 10 obtained under *in situ* circumstances.

In our experiments the excitatory effect of the muscle activity originated from the orthodrom excitation of the motor nerves. However, muscle activity can produce antidromic excitation of the motor nerves, too (Leksell, 1945; Brown, Matthews, 1960; Werner, 1961), and also the sensory nerve can be affected by muscle activity (Granit et al., 1959).

Investigation of the interaction between the nerve fibres revealed that an active fibre could change the threshold for the adjoining inactive one (Blair, Erlanger, 1940; Katz, Schmitt, 1940; Katalymov, 1974). Mathematical study referring to the electric interaction between adjacent nerve fibres was carried out by Clark and Plonsey (1970, 1971) as well as Markin (1970, 1973). Consequently, it seems probable that the muscle could change the excitability of the adjacent nerves in the case when the muscle activity is not strong enough for generating nerve excitation.

Our results and the experiments published by the authors mentioned above support the hypothesis (Biró, 1975) according to which the muscle could exert an additional influence on the mechanism of the regulation taking place in the excitatory processes because the muscles, under some special conditions, could change the excitatory condition of the nerve in the neighbourhood of the muscles.

References

Arvanitaki, A. (1942) J. Neurophysiol. *5* 89
Biró, G. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. *10* 287
Blair, E. A., Erlanger, J. (1940) Amer. J. Physiol. *131* 483
Brown, M. C., Matthews, P. B. C. (1960) J. Physiol. *150* 332
Clark, J. W., Plonsey, R. (1970) Biophys. J. *10* 937
Clark, J. W., Plonsey, R. (1971) Biophys. J. *11* 381
Eccles, J. C., Katz, B., Kuffler, S. W. (1942) J. Neurophysiol. *5* 211
Epstein, R. A., Jackson, S. H. (1970) Appl. Physiol. *28* 407
Granit, R., Pompeiano, O., Waltman, B. (1959) J. Physiol. *147* 399
Hering, E. (1882) Sitzungsberichte der mathematisch-naturwissenschaftlichen Klasse der kaiserlichen Akademie der Wissenschaften *85* 237
Jasper, H. H., Monnier, A. M. (1938) J. Cell Comp. Physiol. *11* 259
Katalymov, L. L. (1974) Physiol. J. USSR *60* 1518 (in Russian)
Katz, B., Schmitt, O. (1940) J. Physiol. *97* 471
Leksell, L. (1945) Acta Physiol. Scand. *10* Suppl. XXXI
Lloyd, D. P. C. (1942) J. Neurophysiol. *5* 153

Markin, V. S. (1970) Biophysics *15* 120 (in Russian)
Markin, V. S. (1973) Biophysics *18* 314 (in Russian)
Masland, R. L., Wigton, R. S. (1940) J. Neurophysiol. *3* 269
Matteucci, C. (1842) cited in: Handbook of Physiology. J. Field (ed.). Section 1: Neurophysiology, American Physiological Society, Washington D. C. 1959, Vol. 1, p. 20.
Rosenblueth, A. (1941) Amer. J. Physiol. *132* 119
Werner, G. (1961) J. Neurophysiol. *24* 401

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Sodium and Potassium Content of Cataractous Human Lenses

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The sodium and potassium content of human senile cataractous lenses was investigated by flame photometry. A parallelism was found between the severity of the senile cataractous disorder and the increase of sodium and decrease of potassium content of lenses. The changes in ion concentration were not entirely continuous; gaps were found for both cations in a certain concentration range.

The involvement of lens proteins in maintaining the gradients for sodium and potassium is also discussed.

Introduction

Recently, a dynamic interaction among the macromolecules, especially proteins, is assumed to be involved in the maintenance of ion gradients in different biological systems (Ling, 1962; Ernst, 1963, 1975; Hazlewood, 1973). In lenses of mammals a gradient was found for several ions without recognizing any membrane function. The potassium concentration is more than 20 times higher and the sodium concentration about 4 times lower in the lenses than in the sera or in the neighbouring aqueous humour (Reddy, Kinsey, 1963; Davson, 1963; Stankiewicz, 1974). It might be considered that the special protein molecules of the lenses are involved in the development of gradients for sodium and potassium.

It seemed interesting to investigate the sodium and potassium content of human lenses under pathological conditions, namely, in different types of senile cataract. In the present study 21 lenses were analyzed for sodium, potassium and water content. A tendency for equilibration of these two ions was found between lenses and the neighbouring media. Although the equilibration was consistent and always ran parallel with the severity of cataract, it was not continuous. A gap was found in changes of both cations that might reflect a physicochemical change of lens proteins. The role of proteins in the maintenance of ion gradients in human lenses is also discussed.

Materials and methods

Cataractous lenses were received from patients with different history and severity of lens opacity. The severity of the cataractous disorder was classified before the operation by slit-lamp examination with maximal mydriasis. The fol-

lowing groups were established: I. Nuclear cataract – the cortex remains clear and nuclear opacity appears yellow-brown. II. Cortical cataract – the nucleus remains clear. III. Complete opaque lens.

Normal lenses were obtained from eyes enucleated because of tumours of the posterior pole. The cataractous lenses with intact capsule were removed by cryo-extraction and used immediately for analyses. The single lenses were dried at 100 °C until constant weight, then ashed at 450 °C for 12 hours. The lenses were always weighed before (wet weight) and after drying (dry weight). The ashes were dissolved in one drop of glacial acetic acid and diluted 100-times with de-ionized water for flame photometrical measurement of sodium and potassium.

Results

In this study a parallelism was found between the cataractous disorders and the changes of sodium and potassium content of the lenses (Table 1). The water content of cataractous lenses was lower than that of the normal ones, but more than 75 per cent water was in lenses characterized by complete opacity (Table 1). In normal lenses the concentration of potassium was about 135 mEq./kg water while the sodium concentration in these lenses was less than 1/4 of it (Table 1). The sodium and potassium content of the lenses characterized by pure nuclear cataract was similar to that of the normal ones. In lenses with cortical cataract the potassium content did not change, but the concentration of sodium increased. The most prominent changes in concentration of both cations were found in lenses with complete opacity (Table 1).

Table 1

K, Na and H_2O content of normal and cataractous human lenses

	Clear lens (3)	Nuclear cataract (5)	Cortical cataract (5)	Complete opaque lens (8)
Na^+ mEq./kg lens water	30.88± 0.96	34.54± 2.35	44.98± 2.20	164.55±8.46
K^+ mEq./kg lens water	135.16±17.61	138.02± 9.36	134.38± 6.40	21.14±1.06
$Na^+ + K^+$ mEq./kg lens water	166.04±16.98	172.56±10.30	179.36± 6.69	185.69±9.08
Wet weight, mg	233.5 ± 5.7	218.4 ± 5.5	203.2 ±10.3	186.7 ±8.3
Dry weight, mg	68.2 ± 0.8	76.3 ± 3.3	65.2 ± 3.5	41.7 ±5.8
Percentage water content of the lens	70.7 ± 1.1	65.0 ± 1.7	67.9 ± 0.5	78.1 ±2.4

Values are expressed as mean ± standard error of the mean
For classification of cataractous lenses see Materials and methods

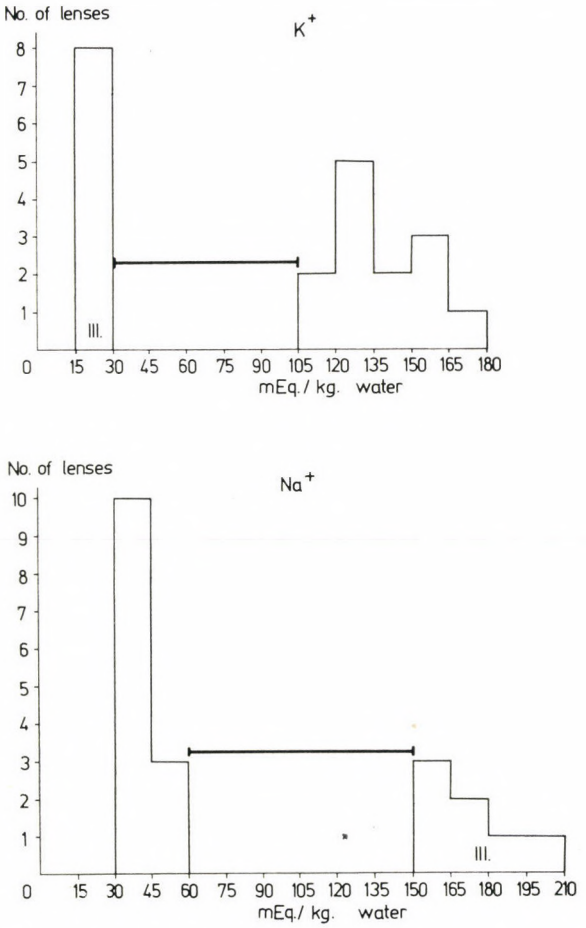

Fig. 1. A frequency histogram of data of potassium and sodium concentration in all of the lenses examined in this study. The marks sign gaps for K and Na, (see Results). The histograms marked by III contain the data of lenses characterized by complete opacity, while the unmarked histograms represent the data of the other groups

These findings show that the severity of cataractous disorders might be characterized by the decrease of potassium and increase of sodium content. Nevertheless the changes of the ion concentration were not entirely continuous. Gaps were found for both cations, i.e. no lenses contained potassium in the range of 30–105 mEq./kg water and only one lens contained sodium in the range of 60–150 mEq./kg water (Fig. 1).

Discussion

In accordance with previous findings also the present study revealed a parallelism between the severity of the senile cataractous disorder and the increase of sodium and decrease of potassium content of lenses (van Heyningen, 1972; Maraini, Mangili, 1973; Maraini, Torcoli, 1974; Stankiewicz, 1974; Duncan, Bushell, 1975).

In the clinical practice the types of senile cataract can well be characterized as follows: nuclear-, cortical-opacity and complete opaque lenses. The classification of cataracts mentioned above signs not only morphological differences but also the changes in the ion composition of lenses. This may be the reason why the ion composition of cataractous lenses has been reinvestigated in the past few years (Maraini, Mangili, 1973; Maraini, Torcoli, 1974; Duncan, Bushell, 1975).

On the other hand, the sodium and potassium measurements suggest that the progress of cataract may not be continuous. The absence of lenses in the range of 30–105 mEq./kg water potassium and 60–150 mEq./kg water sodium may indicate a step-by-step change of the ion composition of the internal milieu in lenses.

Unfortunately, the mechanism and the forces which are maintaining the special ion composition of normal and cataractous lenses are unknown. The integrity of the macromolecules must be of importance, because nowadays different proteins with their fixed charges are supposed to be involved in maintaining ion gradients in biological substances (Ling, 1962; Ernst, 1963, 1975; Hazlewood, 1973). The changes in the inorganic composition of lenses might give an insight into the physicochemical conditions of macromolecules, mainly proteins. Certain alterations of proteins in cataractous lenses have recently been found by fine electron microscopic studies (Maraini et al., 1971; Philipson, 1973; Philipson, Fagerholm, 1973; Gierkowa et al., 1974; Liem-The et al., 1975; Harding, Dilley, 1976). On the basis of experiments concerning the structural changes of proteins in cataractous lenses a relationship must be supposed between the disorders in proteins and alterations of sodium and potassium content revealed in this study. It seems possible that the changes in the sodium and potassium content may indicate certain structural changes of proteins in cataractous lenses.

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References

Davson, H. (1963) The Physiology of the Eye. Little, Brown and Co., Boston
Duncan, G., Bushell, A. R. (1975) Exp. Eye Res. *20* 223
Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest
Ernst, E. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. *10* 95
Gierkowa, A., Sosnierz, M., Bialas, B., Gluza, J. (1974) Klin. Oczna *44* 647

Harding, J. J., Dilley, K. J. (1976) Exp. Eye Res. *22* 1
Hazlewood, C. F. (1973) Ann. N. Y. Acad. Sci. *204* 6
Liem-The, K. N., Stols, A. I. H., Jap, P. H. K., Hoenders, H. J. (1975) Exp. Eye Res. *20* 317
Ling, G. N. (1962) A Physical Theory of the Living State. Blaisdell, Waltham, Mass.
Maraini, G., Carta, A., Pescatori, A., Prosperi, L. (1971) Exp. Eye Res. *11* 83
Maraini, G., Mangili, R. (1973) The Human Lens in Relation to Cataract. CIBA Symposium, Elsevier, Amsterdam
Maraini, G., Torcoli, D. (1974) Ophthal. Res. *6* 197
Philipson, B. (1973) Exp. Eye Res. *16* 23
Philipson, B. T., Fagerholm, P. P. (1973) The Human Lens in Relation to Cataract. CIBA Symposium, Elsevier, Amsterdam
Reddy, D. V. N., Kinsey, V. E. (1963) Arch. Ophthal. N. Y. *63* 715
Stankiewicz, A. (1974) Klin. Oczna *44* 9
van Heyningen, R. (1972) Exp. Eye Res. *13* 155

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Effect of Stretching on the Water-binding of Muscle

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Frog muscles stretched to a length about 1.2 times greater than the original one lost 19 per cent of their water content on the average when kept in normal Ringer solution at a temperature of 2 °C for 24 hours. In $NaHCO_3$-free Ringer solution containing 1.5×10^{-4} per cent lactic acid (pH 6.8) the water content increased by 6 per cent. The change in the weight of non-stretched control muscles taken from the opposite side was insignificant in normal Ringer solution. The increase of water content in Ringer solution containing lactic acid was 22 per cent on the average. The conclusion is drawn that muscle is not an osmotic sack of water-medium and muscle water is bound, a least in part, through swelling.

Introduction

The effect of stretching on the properties of muscle was studied by several authors. Laugier and Benard (1911) loaded the one of a pair of frog gastrocnemius muscle with a weight of 550 g, left the other one unloaded and placed both in distilled water. According to their experiment the weight of the loaded muscle increased by 25 per cent, and that of the unloaded one by 43 per cent. The experiments of Ernst (1926) approximated physiological circumstances better: a stretched frog gastrocnemius (the parallel one was not stretched) took up considerably less water than the non-stretched one in $NaHCO_3$-free Ringer solution containing 0.15 per cent lactic acid. After discontinuing the stretching the water uptake became quicker and some hours later the values of stretched and non-stretched muscles were practically the same.

In the opinion of Ernst et al. (1954) muscles stretched with 15–25 per cent of their resting length are "isotonic" with 0.75 normal Ringer solution, i.e. they keep their weight in 0.75 normal Ringer solution. Furthermore, Ernst and Tigyi (1954) demonstrated that the migration of water was not connected to a parallel migration of ions: non-stretched muscles took up water from 0.75 normal Ringer solution (which had the same concentration of potassium and calcium as normal Ringer solution) and, at the same time, lost 10–15 per cent of potassium and phosphorus. Stretched muscles did not lose water, but lost potassium and phosphorus in 0.75 normal Ringer solution.

The state, "boundedness", structure of biological water is the subject of intensive scientific debates anew (Hazlewood, 1973). Therefore we examined the

effect of stretching and lactic acid on the water-binding of muscle. In order to approximate physiological conditions as near as possible, a $NaHCO_3$-free Ringer solution with 1.5×10^{-4} per cent lactic acid was used.

The changes of water content of stretched and non-stretched muscles was measured in normal Ringer solution and in $NaHCO_3$-free Ringer solution containing lactic acid. We wanted an answer to the question of whether muscle is an osmotic sack (Hill, 1930a, b; Dydynska, Wilkie, 1963), or it is a swollen system (Ernst et al., 1950; Ernst, 1963; Pócsik, 1967; 1969). The experiments proved the latter to be the case.

Methods

Sartorius, gastrocnemius, semimembranosus muscles of frog (*Rana esculenta*) were used, 20 pairs of each. Left and right muscles were stretched alternately, fixed on a glass rod of appropriate form (Fig. 1) with surgical thread and stretched about 1.2 times longer than their resting length. Control muscles were also fixed to muscle stretchers but not stretched. Thereafter, the total mass of the muscle and the glass rod was measured with an accuracy of 1 mg (at the end of the

Fig. 1. Stretching of muscle on a glass rod

experiments the mass of the glass rod and the thread was measured). The muscles were kept in normal Ringer solution or $NaHCO_3$-free Ringer solution of a lactic acid content of 1.5×10^{-4} per cent (1 liter of solution + 1 ml 0.15 per cent lactic acid, pH = 6.8) for 24 hours. The amount of the incubation fluid was large in comparison with the muscle's mass. In order to cause as little biological damage to the muscle as possible the solution was kept in a refrigerator at a temperature of 2 °C. The drops of solution were wiped off the preparations before measurement.

Results and discussion

The data of measurements performed on the different kinds of muscles are shown in Tables 1–3. The tables are divided to four main parts: the data of stretched muscles in normal Ringer solution and in $NaHCO_3$-free Ringer solution containing lactic acid are indicated in the first and second quarters,

Table 1

Data of stretched and non-stretched sartorius muscles in normal Ringer solution and in $NaHCO_3$-free Ringer solution containing lactic acid

1			2			3			4		
Stretched muscles						Non-stretched muscles					
Normal Ringer solution			$NaHCO_3$-free Ringer solution + lactic acid (1 l Ringer solution + 1 ml 0.15 per cent lactic acid)			Normal Ringer solution			$NaHCO_3$-free Ringer solution + lactic acid (1 l Ringer solution + 1 ml 0.15 per cent lactic acid)		
a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)
347	284	−18	253	285	13	363	357	−2	276	354	28
326	296	− 9	317	323	2	337	340	1	311	429	38
280	198	−29	279	290	4	315	309	−2	298	362	21
283	247	−13	332	341	3	270	276	2	301	394	31
300	220	−27	312	321	3	292	298	2	347	414	19
223	159	−29	274	293	7	206	212	3	300	360	20
256	197	−23	272	284	4	196	201	3	311	401	29
268	207	−23	260	267	3	291	298	2	309	379	23
395	321	−19	391	396	1	222	233	5	355	475	34
388	282	−27	349	363	4	358	353	−1	366	457	25
		$\bar{x}_{11} = -22 \pm 7$			$\bar{x}_{12} = 4 \pm 4$			$\bar{x}_{13} = 1 \pm 2$			$\bar{x}_{14} = 27 \pm 6$

a = mass of the muscle at the beginning of the experiment
b = mass of the muscle at the end of the experiment

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Table 2

Data of stretched and non-stretched gastrocnemius muscles in normal Ringer solution and in $NaHCO_3$-free Ringer solution containing lactic acid

1			2			3			4		
Stretched muscles						Non-stretched muscles					
Normal Ringer solution			$NaHCO_3$-free Ringer solution + lactic acid (1 l Ringer solution + 1 ml 0.15 per cent lactic acid)			Normal Ringer solution			$NaHCO_3$-free Ringer solution + lactic acid (1 l Ringer solution + 1 ml 0.15 per cent lactic acid)		
a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)
1229	970	−21	1589	1661	5	1076	1053	−2	1658	2078	25
1390	1196	−14	826	868	5	1389	1389	1	898	1050	17
1943	1629	−16	2104	2163	3	2114	2107	0	2061	2575	25
2177	1794	−18	1306	1399	7	2150	2160	1	1276	1501	18
1398	1147	−18	1491	1557	4	1464	1475	1	1523	1916	26
1353	1082	−20	1189	1285	8	1379	1399	2	1230	1447	18
2047	1655	−19	1009	1086	8	2038	2063	1	996	1230	24
1300	1147	−12	1330	1416	7	1290	1297	1	1336	1546	16
1057	812	−23	1195	1239	4	1036	1046	1	1233	1539	25
1414	1148	−19	755	735	−3	1372	1380	1	767	899	17
		$\bar{x}_{21} = -18 \pm 3$			$\bar{x}_{22} = 5 \pm 3$			$\bar{x}_{23} = 1 \pm 1$			$\bar{x}_{24} = 21 \pm 4$

a = mass of the muscle at the beginning of the experiment
b = mass of the muscle at the end of the experiment

Table 3

Data of stretched and non-stretched semimembranosus muscles in normal Ringer solution and in $NaHCO_3$-free Ringer solution containing lactic acid

1			2			3			4		
Stretched muscles						Non-stretched muscles					
Normal Ringer solution			$NaHCO_3$-free Ringer solution + lactic acid (1 l Ringer solution + 1 ml 0.15 per cent lactic acid)			Normal Ringer solution			$NaHCO_3$-free Ringer solution + lactic acid (1 l Ringer solution + 1 ml 0.15 per cent lactic acid)		
a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)	a (mg)	b (mg)	$x = \frac{b-a}{a} 100$ (per cent)
1350	1198	−11	757	832	10	1379	1399	2	760	908	20
942	736	−22	1265	1382	9	942	952	1	1366	1596	17
874	689	−21	810	855	6	833	842	1	771	954	24
681	564	−17	1052	1098	4	967	987	2	1117	1273	14
2073	1752	−16	843	917	9	1992	2013	1	897	1034	15
1614	1324	−18	725	755	4	1575	1592	1	764	919	20
1832	1520	−17	697	739	6	1918	1899	−1	780	887	14
966	858	−11	887	953	7	866	873	1	809	947	17
781	632	−19	799	854	7	791	793	0	890	1032	16
1075	951	−12	609	669	10	1044	1052	1	814	984	21
		$\bar{x}_{31} = -16 \pm 4$			$\bar{x}_{32} = 7 \pm 2$			$\bar{x}_{33} = 1 \pm 1$			$\bar{x}_{34} = 18 \pm 3$

a = mass of the muscle at the beginning of the experiment
b = mass of the muscle at the end of the experiment

the corresponding data of non-stretched muscles in the third and fourth quarters. Each Table contains the data of 20 stretched and 20 non-stretched muscles. The mass of muscle measured immediately after preparation (stretching) is denoted with "*a*", and that after being kept in the solution with "*b*". The change of water content is also expressed in per cent $\left(x = \frac{b - a}{a} \cdot 100\right)$. Average values ($\bar{x}_{ik}$) and standard deviations (where index $i = 1, 2, 3$ denotes the Table, and index $k = 1, 2, 3, 4$ the corresponding "quarter") are also shown.

Survey of the data presented reveals that stretched muscle loses 19 ± 5 per cent water on the average in normal Ringer solution during 24 hours, while it takes up 6 ± 3 per cent water in $NaHCO_3$-free Ringer solution containing 1.5×10^{-4} per cent lactic acid.

The change of weight of non-stretched muscles in normal Ringer solution is insignificant; in Ringer solution containing 1 ± 1 per cent lactic acid the water content increases by 22 ± 6 per cent.

The question arises why lactic acid is used. Lactic acid is produced in greater amount in stretched than in non-stretched muscle (Ernst, Fricker, 1931). But the effect of lactic acid produced by the muscle is not significant if we compare it with the effect of lactic acid actually present in the solution of great volume.

On the basis of the results the conclusion is drawn that muscle is a swollen system. If muscle were an osmotic sack, its water content would not change in normal Ringer solution with osmotically negligible (1.5×10^{-4} per cent) lactic acid content at 2 °C during 24 hours.

In our opinion the behaviour of stretched muscles can be explained by a decreased water-binding ability, water loss, under the effect of stretching on the one hand (Ernst, 1963) and water uptake from Ringer solution containing lactic acid on the other. The joint effect of these two features has also to be taken into account.

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References

Dydynska, M., Wilkie, D. R. (1963) J. Physiol. *169* 312
Ernst, E. (1926) Pflügers Arch. *213* 131
Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest, pp. 100—133
Ernst, E., Fricker, J. (1931) Pflügers Arch. *228* 700
Ernst, E., Tigyi, J., Zahorcsek, A. (1950) Acta Physiol. Acad. Sci. Hung. *1* 5
Ernst, E., Tigyi, J. (1954) Acta Physiol. Acad. Sci. Hung. *6* 145
Ernst, E., Tigyi, J., Nagy, J. (1954) Acta Physiol. Acad. Sci. Hung. *6* 135
Hazlewood, C. F. (1973) Physicochemical State of Ions and Water in Living Tissues and Model System. Annals of the New York Academy of Sciences, New York, *204* 1—631
Hill, A. V. (1930) Proc. Roy. Soc. B. (London) *106* 445
Hill. A. V. (1930) Proc. Roy. Soc. B. (London) *106* 477
Laugier, H., Benard, H. (1911) J. Physiol. Path. Gen. *13* 497
Pócsik, S. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 147
Pócsik, S. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 395

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Sliding Friction contra Sliding Hypothesis*

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In the problem of muscular activity a very conspicuous one-sidedness prevails today in Huxley's so-called sliding hypothesis, which postpones most of the activity processes to the data of the micro- and submicrostructure. But there are many more important *activity processes:* shortening of the anisotropic bands; widening of the fibre or the whole muscle; volume constriction with action potential and muscle sound; increase in electrical conductivity; the thermocurrent; steady decrease of the force going with increasing shortening in the course of contraction; large and long-lasting contraction due to perfusion with hypotonic solution; disappearance of shortening capability due to hypertonic perfusion (in both cases *single* stimuli); shifts in the inorganic constituents; crystallization of the myosin; increased metabolism in resting muscle during stretching, etc.

Reciting these data I do not intend to discuss how some "theories" could successfully cut free themselves from these experimental facts, notwithstanding I should like to expound one single question entitled: *The Sliding Hypothesis and the Viscous Resistance.*

Speaking concretely I wish to investigate the viscous resistance inside the sarcomere during interdigitation of the thin filaments into the thick ones namely *in relation* to the force exerted by the shortening muscle.

I. Trying to determine the *force of the viscous resistance* I propose to use the formula for the sliding friction

$$F_s = \eta q \frac{\Delta v}{\Delta r}$$

where η = viscosity of the intrafibrillary, i.e. interfilamentous, medium, q = the total sliding surface, and $\frac{\Delta v}{\Delta r}$ = the velocity gradient (v = the velocity of the sliding motion, r = distance between the sliding surfaces; in this case q is very large and r is very small).

* Comment of J. Tigyi's lecture entitled "Biophysics of Muscle Contraction" delivered at the 1st European Expert Committee Meeting on "Perspectives in Biophysics" Budapest, June 2–4, 1976.

1. The value of the *viscosity* of the interfilamentous medium is not known; in case of water $\eta_w \sim 10^{-2}$ P (Poise, $gcm^{-1}s^{-1}$), of glycerol $\eta_g \sim 10$ P; of actin solution $\eta_a \sim 400$ P. Considering these values I propose the surely low value of $\eta \sim 1$ P for the viscosity of the interfilamentous medium.

2. *The sliding surface* (q) of the thin filaments is to be computed. For that reason we start with the following *muscle model:* this contains parallel fibres, has a length of 3 cm, a cross section of 1 cm^2, 20 per cent of which is occupied by interstitial fluid, 80 per cent by fibres. This 80 per cent is 0.8 $cm^2 = 0.8 \times (10^4\ \mu)^2 = 8 \times 10^7\ \mu^2$; the width of a fibre is $50 - 100\ \mu m$, say 70 μm, its cross section ($\pi \times 35^2\ \mu m^2$) $\sim 4000\ \mu m^2$; that means that this *muscle contains* $\sim \frac{8 \times 10^7}{4 \times 10^3} = 2 \times 10^4$ *fibres.*

The cross section of a fibre is occupied by fibrils and sarcoplasm to ~ 50 per cent each; the cross section of a fibril is $\sim 1\ \mu m^2$, accordingly there are $\sim 2 \times 10^3$ *fibrils in a fibre.*

The fibril has the same length as the fibre (or the muscle), i.e. 3 cm; the length of a sarcomere comes to $\sim 3\ \mu m$, thus *the fibril contains* $\sim 10^4$ *sarcomeres.*

The cross section of a sarcomere (= that of the fibril) is $\sim 1\ \mu m^2 = 10^8\ A^2$, that of a thin filament $\pi(25\ A)^2 \sim 2000\ A^2$; the literature assumes that the thin filaments occupy ~ 4 per cent of the cross section of the sarcomere; accordingly, there are $\sim \frac{4 \times 10^6}{2000} = 2 \times 10^3$ *thin filaments in a sarcomere.* Thus there are approximately $2 \times 10^4 \times 2 \times 10^3 \times 10^4 \times 2 \times 10^3 \sim 10^{15}$ *thin filaments* in this muscle model.

The muscle model of 3 cm resting length *shortens* during activity *by* ~ 1 *cm*, i.e. by 1/3 of its resting length; accordingly, a sarcomere shortens by 1 μm. Consequently the *sliding surface* (area) *of 2 facing thin filaments in a sarcomere comes to* $\pi \times 50A \times 10^4 A \sim 10^6 A^2$ and *that of all filaments* in the shortened muscle $q \sim$ $\sim 10^{15} \times 10^6 A^2 = 10^5\ cm^2$ (10 m^2).

3. The *velocity* of the sliding can be determined from the velocity of the shortening in a single sarcomere $\Delta v \sim \frac{1\ \mu m}{50\ ms}$ ($= 20\ \mu m s^{-1}$) $= 2 \times 10^5 A\ s^{-1}$; for the distance Δr $10^2 A$ may be accepted. Thus for the *velocity gradient* this approximate value results:

$$\frac{\Delta v}{\Delta r} \sim \frac{2 \times 10^5 A\ s^{-1}}{10^2\ A} \sim 10^3\ s^{-1}.$$

Consequently *the force of the sliding friction* is

$F_s \sim (1\ gcm^{-1}s^{-1}) \times (10^5 cm^2) \times 10^3 s^{-1}$, i.e.

$F_s \sim 10^8$ dyn at shortening of this muscle model.

II. In contrast to that the so-called *absolute force* for frog muscles comes to $F_m \sim 2$ kpcm^{-2}, $\sim 10^6$ dyn, viz. $F_m < F_s$: *the maximal active force a muscle can exert during contraction is smaller than the force of the sliding friction which should be overcome by the former.*

As to *relaxation* of the shortened muscle there is no doubt that the sliding friction is effective in the same manner as during shortening. Therefore the question arises where the force is taken from to overcome *the viscous resistance opposing the reversal of interdigitation.*

Book Reviews

Principles of Enzyme Kinetics (A. J. Cornish-Bowden). Butterworth and Co., London–Boston, 1976).

The "Principles of Enzyme Kinetics" consists of 10 chapters as follows: 1. Basic principles of chemical kinetics: it deals with the order of reaction, rate constants and transition-state; 2. Introduction to enzyme kinetics; it presents the Michealis–Menten and steady-state mechanisms; 3. How to derive steady-state rate equations; it gives the principle of the King–Altman method and its modifications; 4. Inhibitors and activators; it shows the theoretical treatment and practical possibilities of differentiation of inhibition types; 5. Reaction pathways; it presents the kinetics of the reactions with two substrates; 6. Effects of pH and temperature on enzymes; it deals mostly with reactions with one substrate; 7. Control of enzyme activity; it gives the kinetic treatment of the molecular mechanisms of regulation of metabolic pathways; 8. Analysis of progress curves; it presents the integrated Michaelis–Menten equation and product inhibition; 9. Fast reactions; it deals with transient phase, burst and jump kinetics, and 10. Estimation of rate constants; it shows the practical problems of the determination and calculation of constants.

A great merit of the book is that the core of enzyme kinetics is explained in a simple manner that all biochemists can readily follow. The limitations of the kinetic theory and of the techniques are clearly outlined. It is a well-disciplined book for postgraduate students. However, the chapter on the basic principles of chemical kinetics is rather poor, consecutive and parallel reactions, or autocatalytic reactions are not mentioned. Regrettably the reactions with three substrates are completely absent, although the treatment of those with two substrates is excellent, disregarding the lack of Cleland's method for differentiation of reaction mechanisms by means of product inhibition.

The greatest virtues of this book are the well-presented elements of derivation of steady-state rate equations, the analysis of progress curves and, last but not least, the statistical treatment of results, which in most texts is discussed inadequately or not at all. The book can be highly recommended to research students in enzymology and it will certainly be a useful handbook also for advanced research workers.

T. KELETI

A. Varga (ed.): *Proteinbiosynthese bei Mikroorganismen unter Einwirkung von äußeren elektromagnetischen Feldern* (Fortschritte der experimentellen und theoretischen Biophysik, Bd. 20). VEB Georg Thieme, Leipzig, 1976

The monograph containing 106 pages, 150 references, 34 figures and 23 tables reports on the author's investigations performed on pure *E. coli* (O86) gram-negative and *B. subtilis* (ATCC 6633) gram-positive strains cultured in precisely defined and carefully purified fluid media.

The timeliness of the investigations is supported by the fact that there is a world-wide struggle for the purity of environment nowadays, and "*electromagnetic impurities*", which spread more and more quickly, must

also be mentioned among the *impurities of environment*. Though they are accompanied by small electric and magnetic field strengths it is known that often a slight change of energy of the environment can have a significant effect on the processes going on in the living organism.

In the *theoretical part* of the book (about 40 pages) the author treats the parameters the effect of which must be taken into account as well as their expectable role. The effect of *electric field* is not expectable, because the energy that can be taken up from the environment by a cell is about 10^{18} times smaller than the energy produced by the cell during its activity. On the other hand, an (outer) field strength of some V/cm can be neglected beside the roughly 5×10^4 V/cm in the environment of the membrane. The effect of *electric current* in not expectable either, because the conductivity of fluid medium is 10^8 times greater than that of the membrane; therefore, electric current "flows round" the cell. The rough effect of electrolytic decomposition that may occur here is very disturbing. The absolute value of the energy that can be taken up from *magnetic field* is also small, nevertheless it is several orders of magnitude greater than in the case of electric field; therefore, the effect of magnetic field is worth examining. The factors the effect of which can be taken into account are the dipole moment, nucleus spin, ortho- and para-states and chemical shift. The change of surface energy on the border surface of "cell-fluid medium" can be expected as a result of atomic processes. All these factors are analyzed by the author in detail and in a quantitative way.

The next part of the monograph is constituted by the *experimental part* and the *discussion of results*.

In the experiments the microbe cultures were exposed to the effect of homogeneous, constant or changing fields of a maximum field-strength of 500 G for 1 minute and then cultured at 30°C for several hours. The multiplication was followed photometrically. With careful *pilot experiments* the author proved that magnetic field decreased the time of duplication as determined by the logarithmic law of increase and accelerated the rate of increase by 20—30 per cent. The amount of dry material increased by 50—90 per cent. Similar significant changes occurred also in other microorganisms *(Serratia marcescens, Staphylococcus albus, Proteus vulgaris)*. The parameters necessary for the maximum of relative changes are as follows: magnetic flux = 155 G, optimum frequency = 2 kHz (the 200 kHz found in the book is a misprint), with a great fall at 10 kHz. Treatment with magnetic field (1 s to 1 hour) and changes in temperature (up to 100 °C) are ineffective.

The aim of another series of examinations was to decide whether the effect of magnetic field should *develop directly in the cell or by the mediation of the fluid medium*. (The question is all the more justified, because there are only 1.8×10^{-4} ml of microbe cells in 1 ml of fluid medium.) It was experienced that the primary effect ensued outside the cells. The main point is that the state of energy of the fluid medium changed upon the effect of magnetic field and, therefore, the *electrokinetic parameters* of the microbe cells (electrokinetic potential, the net electric charge of membrane, the energy stored on the surface of effect, etc.) decreased by about 15—30 per cent. As the influence exerted on the fluid medium caused a change inside the cells, the behaviour of cell membrane also had to be examined. It was found that *changes in osmotic pressure* of 3—8 water mm occurred upon the effect of magnetic field. The changes also depended on the field strength and the "age" of the membrane.

Amino acid analysis showed that the composition of amino acid did not change upon the effect of magnetic field; thus, the field did not influence the quality but only the quantity of protein production. According to this the field does not cause real genetical changes.

From the investigations the author draws the *main conclusions* as follows: The acceleration of growth ensuing upon the effect of magnetic field can be attributed to the *decrease of the energy-obstacle (-barrier)* at the border-layer of two phases or partners of reaction. Three mechanisms can be imagined: either an effect similar to chemical catalysis appears, or the flow of material on the membrane is accelerated, or the genetic apparatus is activated. L. SZALAY

Praktische Chemie für Mediziner und Naturwissenschaftler (Henning, H. G., Jugelt, W., Sauer, G.). VEB Verlag Volk und Gesundheit, Berlin, 1976, 434 pages

The recognition that the chemical preliminary training of medical students is a non-negligible part of the whole training gained ground also in the German Democratic Republic after the '50s of this century. An excellent evidence of this recognition is the book reviewed here the title of which became "Praktische Chemie" obviously after a very thorough consideration, since this work takes the middle place between practice in the usual sense and the text-book.

In the preface of the work the authors mention that chemical text-books are not able to suffice the special chemical needs of the training of physicians, and the same is true more or less for practices written for chemists. This consideration led the authors to compile their work of "practical chemistry", the backbone of which is formed by practical exercises in chemistry. The short theoretical material of knowledge that is necessary for the understanding of the exercises is given before and after these practical exercises. Though the book itself cannot replace a more thorough and detailed chemical text-book or a manual in particular, it can serve as an excellent help to anyone who studies the book after having finished some course of chemistry, or — which would be more useful — who actually carries out the practical exercises beside studying the book if there are appropriate laboratory facilities at disposal. The first 40 pages describe the simplest instruments, equipments and methods in the laboratory, not neglecting simple hygienic preventive measures and methods of working of glass shaping.

This is followed by the basic knowledge of general and inorganic chemistry on about 90 pages. This chapter is a little heterogeneous, because it treats the different modes of expression of concentration, their mutual conversions, the basic knowledge about the structure of atoms, theory of chemical bond and a short survey of inorganic chemistry, all demonstrated with practical exercises.

The chapter dealing with the quantitative aspects of chemical reactions occupies about 150 pages. Here one finds description of simple but demonstrative experiments from the field of chemical kinetics, electrolytic dissociation, titrimetry, buffer solutions, electrochemistry, etc., naturally — as mentioned above — with the minimum necessary theoretical support.

After this the basic knowledge of organic chemistry is treated with the same method through about 90 pages. The reviewer cannot conceal his opinion that this chapter is a little crowded. Such a large number of data, relationships and experiments are piled into these 90 pages that it makes the study of this chapter difficult and somewhat tiring as compared with other chapters.

The last, Chapter V, presents the different analytical methods of chemistry. It should be mentioned that in this third, revised edition of the book, which first appeared in 1966, Chapter V contains the largest number of modernizations compared to the two previous editions. Basic elements of photometry, ion-exchange analytics, different methods of chromatography and organic analytics, etc. are included in this chapter.

This valuable work is completed by chemical tables, a four-figure logarithm table, a survey of literature and subject index.

In the reviewer's opinion it would be useful to publish this work in Hungarian.

D. SZABÓ

Biokybernetik, Band V. Materialien des IV. Internationalen Symposiums "Biokybernetik", Leipzig, 19—22 September, 1973 (Ed. H. Drischel, P. Dettmar). VEB Gustav Fischer Verlag, Jena, 1975

In the fifth volume of the well-known series Biokybernetik the material of IV. International Symposium of Biocybernetics is published. The book fits well into the preceding four volumes of the series: it deals with the most timely questions of this modern branch of science at a very high level.

It appears from the book that the organizers of the symposium, who are the editors of the book as well, considered the term biocybernetics in the wider sense of the word. The book treats all the neurophysiological, brain research and psychophysiological

problems, which are important for information processing and cerebral regulation. On 368 pages including 47 lectures of an illustrious and excellent group of authors 6 main spheres of problems are treated. The opening lecture, a very interesting work of Kämmerer from Jena, deals with the approximation of cybernetics from algebra and graph-theory.

The first part of the volume deals with information processing of the central nervous system (six interesting publications). I wish to lay stress on the lecture of the Finnish Bergström whose the subject is the sensomotoric behaviour of the simplified network of neurons.

The second part deals with the question of receptor mechanisms; from this I wish to emphasize the important summarizing work of Grundfest from New-York about peripheral sensory coding.

The third part treats the information processing of the optical system. Of the 10 lectures of this very rich chapter I wish to mention the one of the Soviet team of Fomin et al., who describe the problems of maximum sensitiveness of the model of an optical detector network with the aid of mathematical description.

The fourth part, similarly comprising 10 lectures, deals with the processes of form recognition in the optical system. From this chapter containing a lot of important data the working team of Radilova et al. from Prague can be mentioned: it deals with electroencephalographic correlations of the perceiving of visual patterns.

The fifth part treats a general problem of recognition of patterns. This is the chapter of the book which falls the farthest from the concrete problems of brain physiology. It comprises descriptions of models by mathematicians and experts in system theory.

At last, the lectures of the final, sixth chapter of the book are grouped around the problem of regulation and adaptation. The lecture of Dähnert from Leipzig about the mathematical modelling of the regulation of human system of motion can be mentioned here.

This volume of Biokybernetik is a very important source-book not only for the close circle of experts of this research branch, but also for those who are working in borderland areas of science: physiologists, psychologist, experts of system theory, etc. The form of the volume by Gustav Fischer's Publishing House is also exquisite. Its typography is unobjectionable, the figures are of top quality.

G. ÁDÁM

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FIRST EUROPEAN EXPERT COMMITTEE MEETING ON "PERSPECTIVES IN BIOPHYSICS"

(Budapest, June 2–4, 1976)

The recent development of biological sciences has created such a situation that the biologists of even the richest and most developed countries cannot work without international cooperation.

The demand on a close and multilateral collaboration in biophysics was "in the air" but the necessary activation energy for that was given by professor B. Pullman by proposing a preparatory meeting at Budapest. The sponsor of this meeting was the UNESCO Scientific Cooperation Bureau for Europe headed by professor Jaz. The Hungarian Academy of the Sciences and the Hungarian UNESCO Commission kindly offered their facilities for this purpose.

A group of distinguished scientists from the UNESCO "European and North American Region" accepted our invitation and biophysicist of sixteen countries joined to the 1st European Expert Committee Meeting on "Perspectives in Biophysics", Budapest June 2–4, 1976.

This issue of Acta Biochimica et Biophysica summarizes the content of this meeting.

Most of the papers are published in a form they were presented on the conference according to the manuscript supplied by the authors. A few presentations were summarized by the authors themselves and published in this short form. No corrections were made in the original texts but the obvious misprints has been corrected. The essence of discussions is published in a reported form but the courses of conversation is followed to a certain extent.

Pécs, Dec. 20, 1976

J. TIGYI

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 12 (2), pp. 97—98 (1977)

Introduction at the Opening Session

B. PULLMAN

Institute de Biologie Physico-Chimique, Paris, France

Mr. Secretary General of the Hungarian Academy of Sciences, Mr. Director-General for Science of UNESCO, Ladies and Gentlemen,

I feel particularly privileged to have been entrusted by UNESCO to coorganize with Professors Tigyi and Jaz this 1st meeting of the European Expert Committee on Biophysics and am happy to represent at this organizing committee the French National Committee on Biophysics.

By presiding over the organization of such expert Committees in different branches of modern science UNESCO is undertaking an extremely promising and useful venture. There are a number of obvious advantages in the type of organization which is associated with these meetings. Thus, it becomes evident today that because of the increasing cost of transportation and the simultaneous unfortunate decrease in the funds available from public sources for scientific search in many countries, big international congresses are difficult to organize and manage. The holding of regional meetings, bringing together scientists who geographically live close together becomes a natural tendency in such a situation. Under UNESCO's sponsorship, the European meetings have the extremely important second mission of enhancing scientific collaboration between its two principal groups of developed countries which differ in their political, economical and social structure. The enhancement of collaboration, human relations and friendship between these two groups is a primordial goal of these meetings, and should contribute to the atmosphere of "detente and entente" between East and West which is so essential for the peaceful future and development of mankind. I feel that we must consider ourselves as particularly privileged to be able to contribute here both to the development of science and to the improvement of relations between scientists and nations and I wish to express the profound thanks of the participants to the Scientific Cooperation Bureau for Europe of UNESCO for making this venture possible and to the Hungarian Academy of Sciences for having increased the prestige of this meeting by enabling us to hold it under its auspices, and offering us the hospitality of this beautiful setting.

It will now be our duty to first survey and select the most important and promising directions of research in the field of biophysics. This is a fascinating endeavour. Biophysics is truly one of the advancing frontiers of science which moreover combines to a very high degree refined experimentation and bold theo-

retical thinking. It is thus a privileged field for collaboration between experimentalists and theoreticians. Second, we are in charge of selecting the subjects for possible scientific collaboration between Eastern and Western laboratories and of implementing such a collaboration. The importance of this aspect of our work hardly needs to be underlined. This is the first of a possible series of meetings which should direct and survey the realization of this plan and lead perhaps to a European Organization of Biophysics. Much depends thus on the results of our work here during the next few days. The quality of those who have accepted our invitation and are present at this meeting is such that I, personally, look upon our endeavour with great optimism.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 12 (2), pp. 99–120 (1977)

Neuronal Cooperativity in the Visual System of the Fly

W. REICHARDT

Max-Planck-Institut für Biologische Kybernetik, Tübingen, BRD

Introductory Remarks

The properties of physical, biomolecular as well as the properties of multicellular systems are often analyzed by decomposing them into its subsystems or components. Usually one realizes that these properties cannot be described as random superpositions of the effects of the individual components. Quite the contrary is true; the components cooperate with each other in a well ordered fashion and the behaviour of the system may show characteristic properties and changes which can be described as a transition from disorder to order, or as a transition from one state of order to another state of order.

Complex systems, like the Central Nervous System (CNS) – or even parts of it – possess an enormous number of components. The determination of the detailed behaviour of any individual component is rather hopeless, but fortunately not needed if one is interested in features on a macroscopic scale as for instance the behaviour of an entire organism in its environment. The goal of the investigation is therefore to select the relevant parameters and to neglect every unnecessary information. In this connection the concept of order parameters, well known in phase transition theory, has turned out to be a very useful tool. Equations for such order parameters acquire usually a rather simple structure with respect to their time dependence. The reason for this is that the relaxation time of order parameters is usually much greater than these of the components of the system.

In view of these general considerations, I think there are at least two arguments which arise in investigating the properties of the nervous system: The first one is that a quantitative phenomenological description of the "behaviour" of a nervous system is as necessary as studies of its anatomy and cell physiology. Equally important is a characterization of the functional and computational principles which are involved. The second argument is that the cooperative superposition of local computations or interactions in the nervous system, may lead to a completely new and highly complicated behaviour. Thus, inferences about the system properties, based on knowledge of local computations may be near to impossible.

In this review like paper, a quantitative analysis of the visually induced behaviour of flies is presented. This analysis is an essential prerequisite for an understanding of the basic functional principles underlying transduction and processing of optical information by the nervous system.

At the first level of the analysis, a quantitative phenomenological description of the fly's pattern-induced orientation behaviour is developed. This approach characterizes the basic logical organization of the visual control and processing system used by the fly. From the phenomenological theory, a rather complex behaviour, including orientation towards and tracking of complex patterns, as well as spontaneous pattern discrimination, can be quantitatively predicted. At this level, one does not explicitly take into account the limited neurophysiological knowledge available on the function and structure of the optic ganglia, but rather concentrate on the gross input-output characteristics and their analysis.

At the second level of the analysis, a characterization of the functional principles of those neural subsystems responsible for the gross orientation behaviour is carried out. Essential for this behaviour is that the fly's "open-loop" reaction depends upon the relative position as well as the speed of the optical environment. Types and properties of the neural interactions underlying the "position-" and "motion-dependent" computations can be experimentally specified in terms of a mathematical formalism known as a *Volterra* representation. In this way, one can obtain a functional description of the multi-input (receptors) neural network realized in the fly's visual ganglia. Properties of the neural interactions can be interpreted and connected to behavioural reactions. Thus one obtains a canonical classification of the functional interactions in the network; that is, a conceptualization of the relevant information processing performed by the system. The decomposition of the fly's neural network responsible for visual orientation into a series of functional interactions is conceptual and may have little to do with the actual neuronal circuitry, which would be the subject of the third level of analysis. It has still to be clarified by electrophysiological means whether specific neural interactions in the optic ganglia can be associated with the interaction terms of a Volterra series, or whether other representations – derivable from a rearrangement of the Volterra series – are in more direct correspondence with the actual neuronal components.

Experimental Methods

A freely flying insect possesses six dynamic degrees of freedom: three of translatory and three of rotatory motion. The investigations undertaken so far have each been confined to either one degree of translation (the vertical motion of a horizontally flying fly; Wehrhahn, 1974; Wehrhahn and Reichardt, 1975) or one degree of rotation (the rotatory motion of a horizontally flying fly around its vertical axis; Reichardt, 1973). Measurements were carried out by means of highly sensitive, fast mechanoelectric transducers, which sense either the lift force or the flight torque generated by a test fly. Their use guarantees controlled flight conditions. When a contrasted optical environment is moved in front of a fixed flying test fly, one is operating under so-called "open-loop" conditions, whereas when the transducer signal is used to control the position and speed of the environment by simulating the flight dynamics, the conditions are called "closed-loop".

In this paper, I shall confine myself to the behaviour of the fly around its vertical axis (rotatory degree of freedom). Many of the results, as well as their theoretical interpretations, have more recently been verified also in one of the translatory degrees of freedom: the vertical motion of a fly in horizontal flight. Most of the experiments were carried out with female houseflies *Musca domestica* (head rigidly fixed to the thorax), some of the results reported here refer to the fruitfly *Drosophila melanogaster*, and a few to the beetle *Chlorophanus viridis*.

A Phenomenological Approach to Flight Orientation Behaviour

Flight Dynamics

The results from measurements on fixed flying flies, using fast torque-compensation techniques (Reichardt, 1973), and the comparison of these results (Land, Collett, 1974) with the behaviour of freely flying flies have led to the conclusion that the aerodynamics of flight for rotation of a fly around its vertical axis can be described in good approximation by the following ordinary differential equation:

$$\Theta\ddot{\psi}(t) + k\dot{\psi}(t) = -F(t). \tag{1}$$

where $\Theta = 1.5 \times 10^{-3}$ g cm^2 designates the moment of inertia around the vertical axis of the fly, $k \approx 0.2$ g cm^2 s^{-2}, the friction between the air and the wings under rotation, and F, the flight torque around the fly's vertical axis. In the experimental setup, ψ designates the angle between the direction of flight and a defined point on a cylindrical panorama, the axis of which conforms with the vertical axis of a fixed flying test fly suspended by the flight torque compensator.

In order to simulate free flight conditions, the left-hand side of Equation (1) is computed on line for closed-loop experimentation by an analog electronic device. The basic setup used in these experiments is shown in Figure 1.

Spontaneous Behaviour

In a no-contrasted homogeneously illuminated environment a fly flies in all directions with equal probability. When these conditions are simulated in the experimental setup of Figure 1 the fly's torque signal turns out to be a stationary, zero mean, stochastic signal $N(t)$ with a gaussian density distribution and an exponential autocorrelation $S(\tau) = Ae^{-\gamma|\tau|}$. Due to the gaussianity, the process $N(t)$ is completely and quantitatively characterized by its autocorrelation (Poggio, Reichardt, 1973a). Without visual contrast stimuli the fly spontaneously "searches around", apparently in a random (but correlated) manner. Interestingly, a small, black object which does not move relative to the fly's retina does not affect the torque signal of the fly. Thus, stabilized retinal images of this kind do not apparently influence the orientation behaviour of the fly.

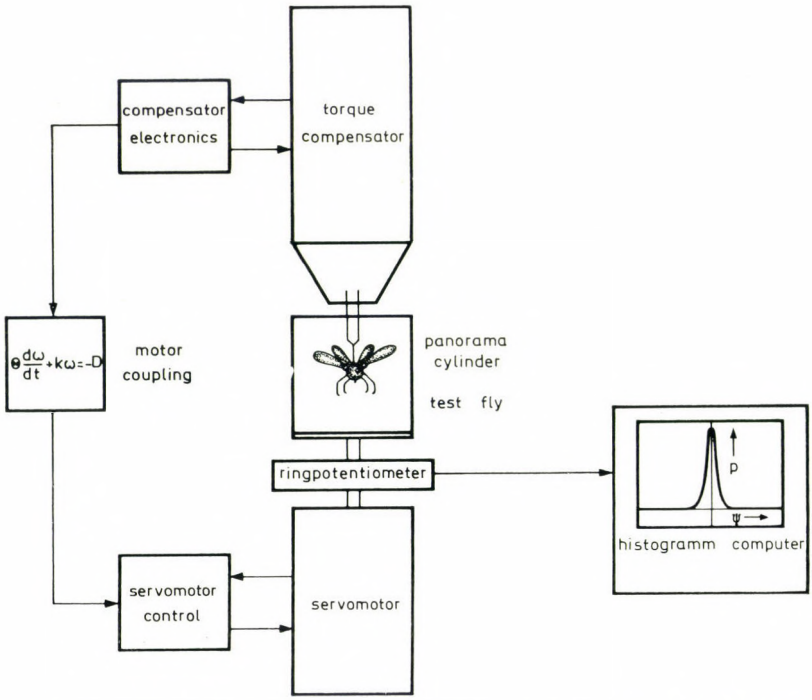

Fig. 1. Simplified diagram of the experimental setup (closed-loop system). A test fly suspended from the torque compensator is able to control the velocity of a patterned panorama by its own torque signal. The transfer properties of the compensator, the motor coupling block and the servomotor give an approximation of the dynamics of the equivalent free flight conditions. The instantaneous position of the panorama is signaled by a ring potentiometer and evaluated by a computer. For more details, see the text

Orientation towards an Object in the Environment

Induced torque responses are observed whenever a contrasted optical environment is moved relative to the fly's compound eyes.

Elementary orientation behaviour is induced under closed-loop conditions when the optical environment consists of a single, vertically oriented stripe or stripe segment placed in front of a white, homogeneously illuminated background. When a test fly suspended by the torque compensator is coupled to this environment, the white panorama cylinder carrying the black stripe is rotated by the fly until the stripe has reached the position $\psi = 0$; this position is defined by the fly's direction of flight. The object (stripe) fluctuates around its stable position with a gaussian-like angular distribution, as shown in Figure 2 in the form of a position histogram. This is the only stable equilibrium position for an environment consisting of a single black stripe. In the equivalent free flying situation, the

fly would be free to orient on its horizontal plane towards the stationary object, and the histogram would show the fraction of time the fly gazed at any part of the stationary environment.

Due to the fact that stabilized retinal images do not elicit a torque response, the result of the one-stripe experiment leads to the conclusion that the visual system derives position information from relative motions between the environment and the compound eye.

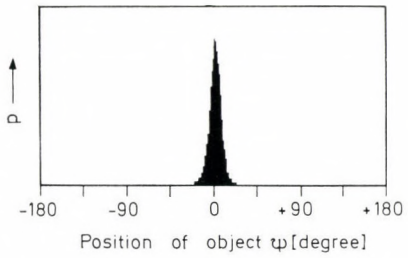

Fig. 2. Position histogram of a black, vertically oriented stripe, recorded during stationary fixation of the fly: p, position probability; ψ, angular coordinate

Let us now designate the induced torque response to moving stimuli under open-loop conditions by the functional $R\{\psi(t), \dot{\psi}(t)\}$. Since the fly's reaction under normal object fixation mainly depends upon the instantaneous values of ψ and $\dot{\psi}$, R reduces to a function of ψ and $\dot{\psi}$, which can be written as

$$R(\psi, \dot{\psi}) = T(\psi, \dot{\psi}) + \rho(\psi, \dot{\psi}), \tag{2}$$

where T represents its even (symmetric) and ρ its odd (antisymmetric) part in $\dot{\psi}$. It has been shown experimentally that Equation (2) can be approximated by a linear expression in $\dot{\psi}$ which reads

$$R = +D(\psi) + r(\psi)\dot{\psi} \tag{3a}$$

or

$$R = +\frac{\partial}{\partial \psi} U(\psi) + r(\psi)\dot{\psi}\,, \tag{3b}$$

where U is the "potential" associated with D (Poggio, Reichardt, 1973a).

It should be pointed out here that the description of R is based on a "quasi-stationary" phenomenological approximation. This simplification is the basic reason why Equation (3) is valid only for $\dot{\psi}^2 > 0$.

Figures 3 and 4 contain the results of an experimental determination of $D(\psi)$, $U(\psi)$ and $r(\psi)$ with a single black stripe on a white background environment. $D(\psi)$ associated with the single-stripe pattern is an odd function, with zero crossings at $\psi = 0°$ and $\psi = \pm 180°$; $U(\psi)$ is the corresponding "potential". As shown in Figure 4, $r(\psi)$ can be approximated by $r(\psi) = r_0 = \text{constant}$. The stable equi-

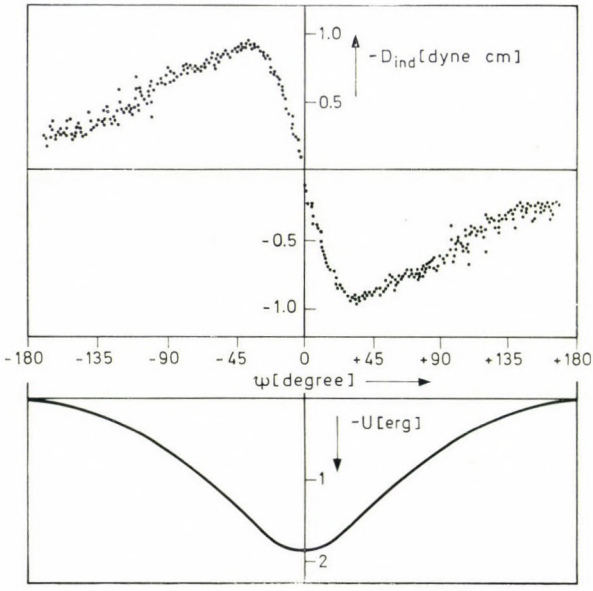

Fig. 3. The direction-insensitive reaction component d_{ind} (ψ) (attractiveness), induced by a vertical black stripe randomly oscillated with small amplitude around each given ψ position (top) and the corresponding potential $U(\psi)$ (bottom). $D(\psi)$ and $U(\psi)$ are averages from 111 individual measurements

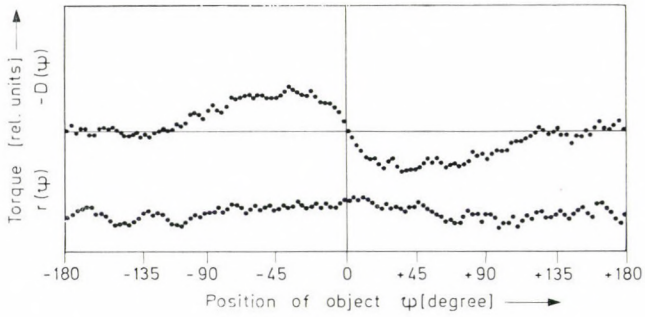

Fig. 4. $D(\psi)$ and $r(\psi)$ generated by a black stripe segment (5° wide, 12.5° long) presented to the lower parts (below the equator) of the compound eyes. The stripe segment was rotated with constant speed (8°/s), and the measured reaction was decomposed into the direction-insensitive component $D(\psi)$ and the direction-sensitive one, $r(\psi)$. $D(\psi)$ disappears where only the upper parts of the compound eyes are stimulated

librium point where stationary fixation takes place is located at $\psi = 0$; here the potential $U(\psi)$ has its minimum. Near the equilibrium, $D(\psi)$ can be approximated by $D(\psi) = \alpha\psi$, so that Equation (3a) takes the linear form

$$R(\psi, \dot{\psi}) \approx \alpha\psi + r_0\dot{\psi}. \tag{4}$$

If we now turn from the open-loop to the closed-loop experimental conditions, we can write Equation (1) as follows:

$$\Theta\ddot{\psi}(t) + k\dot{\psi}(t) = N(t) - R[\psi(t), \dot{\psi}(t)]. \tag{5}$$

The right-hand side of Equation (5) is formulated under the assumption that the nerve correlates of N and R add in the CNS of the fly. This assumption has been critically tested in the neighborhood of the fixation equilibrium and found to be valid (Poggio, Reichardt, 1973a).

Linear Phenomenological Approach to Single-stripe Fixation

As mentioned before, near the fixation equilibrium the equation of motion can be linearized;

$$\Theta\ddot{\psi}(t) + k\dot{\psi}(t) + N(t) - \alpha\psi(t) - r_0\dot{\psi}(t). \tag{6}$$

In this notation, as in Equation (5), we neglect the delay $\varepsilon < 30$ ms between stimulus and response, which can be justified on theoretical grounds. The stationary solution of Equation (6) is given by the stationary distribution $p(\psi)$:

$$p(\psi) = \sqrt{\frac{1}{2\pi\sigma^2}}\, e^{-(\psi^2/2\sigma^2)}. \tag{7}$$

The standard deviation σ in $p(\psi)$ depends upon the parameters Θ, k, α, r_0, and γ. Since σ^2 is given by the autocorrelation $S_{\psi,\psi}(\tau)$ of ψ at $\tau = 0$, one arrives at

$$\sigma^2 = \frac{A}{\Theta^2\, a\, b} \cdot \frac{b + \gamma}{(a + b\gamma + \gamma^2)}, \tag{8}$$

where $a = \alpha/\Theta$ and $b = (k/\Theta) + (r_0/\Theta)$. σ^2 critically depends upon A and b. It is proportional to A, the average fluctuation power of N, and inversely proportional to α, the steepness of $D(\psi)$ in the neighbourhood of $\psi = 0$, and to b, which contains the two "friction" parameters k and r_0. In this connection, the role of r_0, the coefficient of the direction-sensitive contribution of the induced reaction $r_0\dot{\psi}$, is of special interest. If r_0 associated with a given pattern is increased, leaving α constant, the quality of the stationary fixation, expressed by $1/\sigma$, should improve, a prediction that has been experimentally tested and verified (Poggio, Reichardt, 1973a).

Tracking of a Single Object

Equation (6) allows us to predict the orientation behaviour under tracking tasks. For instance, a free flight situation in which a black stripe moves at great distance from the animal with constant angular velocity $\pm\omega$ can be described by adding the term $\pm k\omega$ to the right side of Equation (6). Solving Equation (6) under this additional condition does not result in a change of σ in $p(\psi)$, but in a shift of the argument by $\pm(k/\alpha)\omega$, so that a new distribution is given by $p(\psi \pm \pm(k/\alpha)\omega)$. A freely flying fly should follow the stripe with the same angular velocity $\pm\omega$ and with an angular lag

$$\psi_{tr} = \pm(k/\alpha)\,\omega. \tag{9}$$

Stability is restricted to the linear range of $D(\psi)$ in the neighbourhood of $\psi = 0$. Hence, under this condition, stable tracking should be observable for tracking angles of about $-20° < \psi_{tr} < +20°$. These predictions, which only hold for constant angular object velocity, have been experimentally investigated and found to be in full agreement with the predictions based on Equation (6).

If the object is embedded in a contrasted random dot pattern and rigidly connected to it the description remains essentially the same. The only difference is that the parameter r and α are here different since they depend on the contrast of the background texture, which "masks" the object. For equal object and background contrasts α equals zero: the object is not "seen" by the fly. However, if "incoherent" relative motion takes place between object and background texture, the "masking" inhibition is destroyed and the parameter α, which is a measure of the attractiveness of the object, takes a value characteristic of the no-texture situation. This observation appears as a consequence of the functional properties of the nervous interactions which compute the object attractiveness. The relevant point is that, at this phenomenological level, it is possible to describe tracking of an object moving at constant speed in front of a contrasted texture. If one solves Equation (6) for these conditions one arrives at an angular lag of

$$\psi_{tr} = \frac{k}{\alpha}\left(1 + \frac{r_1}{k}\right)\omega \tag{10}$$

where r_1 is associated to the direction sensitive optomotor response elicited by the random dot background pattern (Virsik, Reichardt, 1974). The fly and the object move with an average angular velocity ω with respect to the background pattern. Thus, ψ_{tr} and ω are proportional to one another as in Equation (9) but with a larger proportionality factor. An interesting sideline to this is that if the coupling of the fly to the background is artificially inverted so that the background texture rotates in the same direction of the target with twice its average speed, Equation (10) becomes

$$\psi_{tr} = \frac{k}{\alpha}\left(1 - \frac{r_1}{k}\right)\omega \tag{11}$$

Equation (11) predicts that the tracking angle decreases and eventually changes its sign when $(r_1/k) > 1$. In this case, the angular lag during tracking becomes an angular lead. Equations (9) through (11) have been tested in a series of experiments (Virsik, Reichardt, 1976) and to be found in complete agreement with the theoretical predictions. They lead to the conclusion that the moving object is perceived and tracked in linear independence upon the background. Figure 5 illustrates the case in which the texture contrast amounts to $m = 55$ per cent.

A More Complex Environment

If the complexity of the environment is increased – for instance by replacing the single stripe with a two- or multistripe environment – stationary orientation behaviour is observed which in general cannot be derived simply from the behaviour observed in single-stripe experiments. However, the question whether complex behaviour can be understood in terms of elementary behaviour can be formulated in the following way: Can one construct a complex $D(\psi)$ function associated to a two- or multistripe environment from the single-stripe $D(\psi)$ by shift and superposition? This question has been tested, in particular with a two-stripe environment. For angular stripe separations between $0° < \psi \leq 40°$, one observes – as in the case of a single-stripe environment – only one orientation equilibrium; this is located at the symmetry line between the two stripes. This observation is not a trivial one, as the average optical resolution of the Musca compound eye amounts to about 2°. For larger angular separations ($40° < \Delta\psi < 180°$), two stable orientation equilibria are observed; they are located in the neighborhood of the two stripes. If we designate with $\Delta\psi^*$ the angular distance between these stable equilibria, then $\Delta\psi^* \leq \Delta\psi$ for $\Delta\psi > 40°$; $\Delta\psi^* = \Delta\psi$ only holds for $\Delta\psi = 180°$. The orientation of the fly in either one of the two equilibrium directions has equal probability. In Figure 6, the orientation probability distribution $p(\psi)$ and the corresponding $U(\psi)$ functions are plotted for the two cases $\Delta\psi = 20°$ and 80°. The number of maxima in $p(\psi)$ corresponds to the number of minima in $U(\psi)$. Concerning the question raised before, one can easily show that $U(\psi)$ for $\Delta\psi = 80°$ can be calculated from a single-stripe $U(\psi)$ by shift and superposition, hence $U(\psi)$ for $\Delta\psi = 80°$ equals $U(\psi) - 40°) + U(\psi + 40°)$. The same holds for separation angles $\Delta\psi > 80°$. Quite different, however, is the situation for separation angles $0 < \Delta\psi < 80°$. This is shown by the example $\Delta\psi = 20°$ in Figure 6. Comparing the double-stripe potential for $\Delta\psi = 20°$ with the one generated by the single stripe, it has to be concluded that the first one cannot be derived from a superposition of the responses produced by each stripe independently. This observation was made in the entire range of $0 < \Delta\psi < 80°$; the discrepancy, however, decreases with increasing $\Delta\psi$. Therefore for double-stripe environments and separation angles $0 < \Delta\psi < 80°$, the superposition principle holds only qualitatively but not quantitatively. A tentative explanation is that the effects induced by each of the two stripes mutually inhibit each other; the inhibition range, however, does not extend remarkedly beyond separation angles $\Delta\psi \pm 80°$ (Reichardt, Poggio, 1975).

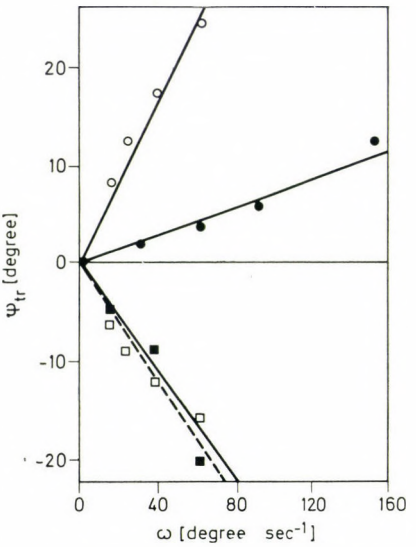

Fig. 5. Tracking of a black vertical stripe moving with constant angular speed ω in front of a background (360°) around the fly. The fly tracks the stripe with the same angular velocity and a mean angular lag ψ_{tr} as shown in the figure as a function of stripe velocity ω. The mean values plotted here were obtained from one fly. • • • = Tracking of the stripe in front of a white background. ○ ○ ○ = Tracking of the stripe in front to a random dot texture (360° angular extension) with an average contrast $m = 55$ per cent. ■ ■ ■ = Tracking of the stripe in front of the same random texture background but with reversed coupling of the fly to the background. □ □ □ = Predicted from the data • • • and ○ ○ ○. The full lines (the dotted line corresponds to the points □ □ □) are regression lines

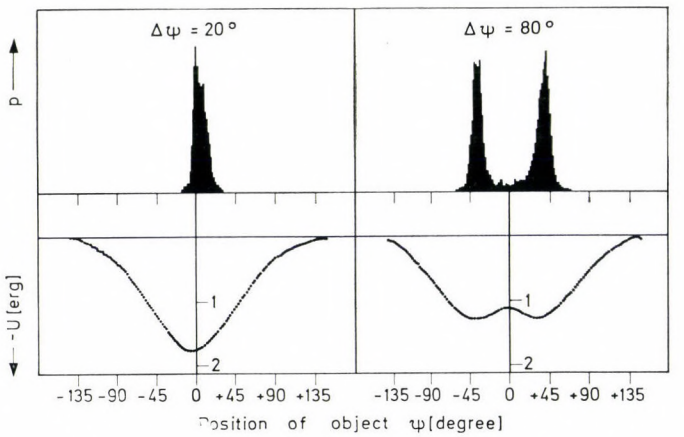

Fig. 6. (Top) Position histrograms for two-stripe patterns (stripe separation angle $\Delta\psi = 20°$ and $\Delta\psi = 80°$) recorded during the stationary phase of orientation. (Bottom) Corresponding experimentally measured potential functions $U(\psi)$

Nonlinear Theory of the Pattern-Induced Flight Orientation

The stationary solution of Equation (5) was confined to the regions near the equilibrium states where a linear approximation golds. In general, $D(\psi)$ and correspondingly, $U(\psi)$ are nonlinear functions associated to the surrounding pattern. A general solution of Equation (5) for arbitrary $D(\psi)$ and $U(\psi)$ therefore constitutes a nonlinear problem. Equations of the type

$$\Theta\ddot{\psi} + [k + r(\psi)]\dot{\psi} + \frac{\partial U(\psi)}{\partial \psi} = N(t)$$

$$\text{with } U(\psi) = U(\psi + 2\pi n) \qquad n = 0, 1 \ldots \tag{12}$$

where $N(t)$ is a noise process, are known as Langevin equations. To solve the stochastic Equation (12) is equivalent to characterize completely the statistics of the random process $\psi(t)$. An exact solution is formally possible on the basis of the theory of Markov processes (Stratonovitch, 1968; Skorokhod, 1965). The statistics of $\psi(t)$ can be given by the Fokker-Planck technique which associates with Equation (12) a partial differential equation in the instantaneous probability density of ψ. The derivation of the solutions is treated in detail by Poggio and Reichardt (1973a) and by Reichardt and Poggio (1975).

To simplify here the treatment, we make the assumption that Θ is negligible, since its value is (in the fly *Musca*) very small. Moreover, it is assumed that the friction parameter associated to a given pattern is a constant, independent from $\psi : r(\psi) = r_0$. If the random process $N(t) = W(t)$ would be gaussian and "white", $\psi(t)$ would then be a Markov process defined by

$$\dot{\psi} + \frac{1}{k + r_0}\frac{\partial U(\psi)}{\partial \psi} = \frac{W(t)}{k + r_0} \tag{13}$$

where $W(t)$ would be "white" noise with a spectral density c. In the more realistic case of $N(t)$ being a "coloured" gaussian process with an e-type first order autocorrelation, Equation (12), written in the phase space, takes the form

$$\dot{\psi} + \frac{1}{k + r_0}\frac{\partial U(\psi)}{\partial \psi} = \frac{N(t)}{k + r_0} \tag{14}$$

$$\dot{N} + \gamma N = W(t)$$

W has the spectral density $(A\gamma)$.

While a Fokker-Planck equation can be easily derived for Equation (13) as well as for Equation (14), its stationary solution, simple in the "white" noise case, becomes difficult in the realistic case of Equation (14). I shall at first briefly consider the "white" noise Equation (13) as the associated analytic solutions are illustrative. Moreover, the "white" noise assumption describes well fixation of patterns whose associated potential is "small" compared to the associated friction r_0, as in the case of large structured panoramas.

The Fokker-Planck equation in the transition probability distribution function $p(\psi; t)$ associated to Equation (13) is

$$\frac{\partial}{\partial t} p(\psi; t) = + \frac{\partial}{\partial \psi} \frac{1}{k + r_0} \frac{\partial U(\psi)}{\partial \psi} p(\psi; t) + \frac{c}{(k + r_0)^2} \frac{\partial^2}{\partial \psi^2} p(\psi; t) \qquad (15)$$

Its stationary solution (for $t \to \infty$) can be derived from Equation (15) and the appropriate boundary conditions as

$$p(\psi) = ce^{-U(\psi) \frac{k+r_0}{c}} . \qquad (16)$$

Equation (16) relates an arbitrary nonlinear (cyclic) potential $U(\psi)$, associated to a given pattern, to the stationary "error angle" distribution $p(\psi)$. Consequently, once position and movement response [$D(\psi)$ and $r_0\dot{\psi}$] associated to a pattern are known, the spontaneous orientation behaviour of the fly is characterized in its stationary state by the stationary solution of the Fokker-Planck equation.

The mapping defined by Equation (16) describes a one-to-one correspondence between potential profile and $p(\psi)$. A large value of c (spectral density of the fluctuation process) maps the entire potential with equal weight. Small values of c, however, result in a mapping of practically only the minima of $U(\psi)$ into $p(\psi)$. Thus, the nonlinear cooperative superposition of simple, local computations determines a nontrivial pattern orientation behaviour and underlies an elementary "classification" of patterns according to their order parameters. Numbers and positions of the minima can be considered as order parameters distinguishing different classes of patterns. As we have seen before, the potential of two or more objects show "symmetry breakings" with respect to the potential minima. Concepts like phase transitions can be applied in the cases to the fly's pattern discrimination behaviour (Poggio, Reichardt, 1973a).

The considerations, valid under the "white" noise hypothesis, essentially apply also to Equation (14). Since this equation and the associated Fokker-Planck equations do not satisfy the condition of detailed balance (Graham, Haken, 1971), no general method is available to obtain the stationary distributions. An approximative solution (Reichardt, Poggio, 1975) however illustrates the role played by the coloured spectrum. This solution is given by

$$p(\psi) \propto \exp\left\{-\frac{\gamma}{A}(k + r_0)U(\psi) - \frac{1}{2A}[D(\psi)]^2 + \frac{1}{\gamma(k + r_0)} \frac{dD(\psi)}{d\psi}\right\} \qquad (17)$$

and clearly shows that $p(\psi)$ may contain two maxima, even if $U(\psi)$ has only one minimum. Due to the non-white spectrum of the fluctuations an "early symmetry breaking" in the peaks of the probability distribution can take place without a corresponding symmetry breaking in the potential minima, an observation which has been made in connection with the two-stripe experiments. The effect illustrates the importance of the nature of the fluctuations, when not thermal-like, in determining the phase transition behaviour of a system.

Towards the Underlying Neural Interactions

The phenomenological theory of the pattern-induced flight orientation, as summarized above, characterizes the basic logical organization of the orientation behaviour at the level of the responsible neural interactions. However, the phenomenological theory does not enable us to specify these interactions because the variables ψ and $\dot{\psi}$ are only indirectly related to the organization of the receptor inputs and to the interaction processes in the CNS. Therefore one needs a description at a deeper level by which receptor organization and neural interactions can be conceptually taken into account.

In the following, the general properties of nonlinear systems with n inputs (receptors) and one output (reaction) will be characterized and compared with the results obtained at the phenomenological level.

In relating the two levels, we may confine ourselves to the open-loop reaction $R(\psi, \dot{\psi})$, as every behaviour observed so far under closed-loop conditions can be derived from the behaviour studied under open-loop conditions. The phenomenological Equation (5), which takes into account the dynamics of flight, links (open-loop) information processing in the visual input by the nervous system as independent from the motor loop being "open" or "closed". The observations suggest that an analysis of the computations performed under open-loop conditions, as it is very often the case, is in fact completely sufficient for an understanding of the behaviour.

A Volterra Description for Systems with n Inputs and One Output

Let us consider a class of systems with n inputs and one output. A rather general representation of this class can be given by an extension of the Volterra series to multi-input systems. This extension is given by

$$\begin{aligned} y(t) = g_0 &+ \sum_{j=1}^{\infty} \sum_{i_1, \ldots, i_j}^{n} \int_{-\infty}^{+\infty} \ldots \int_{-\infty}^{+\infty} \mathrm{d}\tau_1 \ldots \mathrm{d}\tau_j \prod_{r=1}^{j} x_{i_r}(t - \tau_1) \\ &\cdot g_{i_1, \ldots, i_j}(\tau_1 \ldots \tau_j) \\ &= g_0 + \sum_{i=1}^{n} \int_{-\infty}^{+\infty} \mathrm{d}\tau_1 x_i(t - \tau_1)\, g_i(\tau_1) + \\ &+ \sum_{i,j}^{n} \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} \mathrm{d}\tau_1\, \mathrm{d}\tau_2 x_i(t - \tau_1)\, x_j(t - \tau_2)\, g_{ij}(\tau_1, \tau_2) \\ &+ \ldots . \end{aligned} \tag{18}$$

In Equation (18) the $x_i(t)$ represent the input signals and $y(t)$ the output of a multi-input, nonlinear system. The first-order kernels correspond to the impulse re-

sponses of linear systems, whereas higher order kernels characterize the nonlinear properties of self- and cross-interactions. Systems describable by Equation (18) are time invariant, have finite memory, and their input-output variables are bounded.

Equation (18) is an infinite series whose complexity increases rapidly with j (the order of nonlinearity) and n (the number of inputs). In the functional description of many biological systems, it is often possible to neglect terms of orders higher than the second (Marmarelis, McCann, 1973). In this case, the decomposition (schematized in Fig. 7) is possible (Poggio, Reichardt, 1973b): Expressed in words, if an n-input system has nonlinearities up to the second order, it is equivalent to the sum of $\binom{n}{2}$ two-input systems (non-linear up to the second order,) which are all the possible combinations of the n channels, two by two. Generalizations to higher order nonlinearities are immediate. If an n-input system has nonlinearities up to the jth order ($n \geq j$), it can be decomposed into the linear sum of j-input systems. In particular, in studying n-input systems with nonlinearities up to the second order, it is sufficient to consider two-input systems with nonlinearities up to the same order. The functional properties of these two-input systems are represented by

$$\begin{aligned} y(t) = g_0 &+ \int_{-\infty}^{+\infty} g_1(\tau)\, x_1(t-\tau)\, \mathrm{d}\tau + \int_{-\infty}^{+\infty} g_2(\tau)\, x_2(t-\tau)\, d\tau \\ &+ \int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty} \mathrm{d}\tau_1\, \mathrm{d}\tau_2 g_{11}(\tau_1, \tau_2)\, x_1(t-\tau_1)\, x_1(t-\tau_2) \\ &+ \int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty} \mathrm{d}\tau_1\, \mathrm{d}\tau_2 g_{22}(\tau_1, \tau_2)\, x_2(t-\tau_1)\, x_2(t-\tau_2) \\ &+ \int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty} \mathrm{d}\tau_1\, \mathrm{d}\tau_2 g_{12}^{*}(\tau_1, \tau_2)\, x_1(t-\tau_1)\, x_2(t-\tau_2)\,. \end{aligned} \tag{19}$$

where $g_{12}^{*}(\tau_1, \tau_2) = g_{12}(\tau, \tau_2) + g_{21}(\tau_2, \tau_1)$. A graphical description of Equation (19) is presented in Figure 8. Graph (*a*) represents a first-order (linear) term, (*b*) a second-order self-interaction, and (*c*) a second-order cross-interaction. It is always possible to split (*c*) into two parts, a symmetric (*S*) one (*d*) and an antisymmetric (*A*) one (*e*).

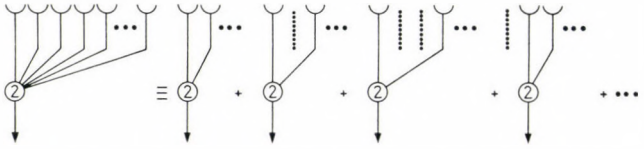

Fig. 7. Schematic representation of the decomposition property for Volterra systems with nonlinearities up to the second order

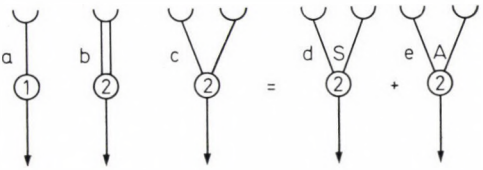

Fig. 8. Schematic decomposition of two-input Volterra systems with nonlinearities up to the second order: (a) Linear term; (b) self-interaction term; (c) cross-interaction term; (d) symmetric (even) part of cross-interaction term; (e) antisymmetric (odd) part of cross-interaction term

An Application of the Volterra Formalism to the Flight Orientation Behaviour

The perception of motion plays an important role in the behaviour of the fly. It has been shown that the stimulation of two receptors of a compound eye forms the necessary and sufficient requirement for eliciting a direction-sensitive optomotor reaction (Kirschfeld, 1972). Investigations of this reaction's dependence upon the contrast, structure and velocity of a moving pattern have led to the conclusion that the associated nonlinear interaction processes in the fly's CNS are of no higher order than the second. It seems therefore at first reasonable to consider in Equation (18) only up to second-order terms and their decomposition, corresponding to the representation given in Figures 7 and 8 and their mathematical formulation according to Equation (19).

The time average $\overline{y(t)}$ of the output is easy to measure under open-loop conditions. If only up to second-order nonlinear interactions are taken into account, this time average, as derived from Equation (19), is given by

$$\overline{y(t)} = g_0 + \overline{g_1 x_1} + \overline{g_2 x_2} + \int_{-\infty}^{+\infty} w_{11}(\tau)\, \Phi_{11}(\tau)\, \mathrm{d}\tau + \int_{-\infty}^{+\infty} w_{22}(\tau)\, \Phi_{22}(\tau)\, \mathrm{d}\tau + \int_{-\infty}^{+\infty} w_{12}(\tau)\, \Phi_{12}(\tau)\, \mathrm{d}\tau\,, \tag{20}$$

where

$$w_{11}(\tau) = \int_{-\infty}^{+\infty} \mathrm{d}\tau_2 g_{11}(\tau_2 + \tau_1, \tau_2)$$

and

$$\Phi_{11}(\tau) = \lim_{T \to \infty} \frac{1}{2T} \int_{-T}^{+T} x_1(t)\, x_1(t + \tau)\, \mathrm{d}t\,.$$

Corresponding expression hold for w_{22}, w_{12}, Φ_{22} and Φ_{12}. Φ_{11} and Φ_{22} are even (symmetric) functions in τ as they represent autocorrelations of x_1 and of x_2. However, the cross-correlation function Φ_{12} is, in general, neither even nor odd. Corresponding remarks are valid for w_{11}, w_{22} and w_{12}.

From the phenomenological analysis of the fly's orientation behaviour, it followed that the reactions induced under open-loop conditions can be split into two parts: $T(\psi, \dot{\psi})$, the direction-insensitive motion, and $\rho(\psi, \dot{\psi})$, the direction-sensitive motion.

A corresponding decomposition into direction-insensitive and direction-sensitive average responses is possible by splitting the reduced kernels w into even and odd parts. Terms of zero order, linear terms and self-interaction terms, such as the even term of the cross-interaction, are direction insensitive. Only the odd terms of the cross-interaction are direction sensitive. Separating the even (symmetric) and odd (antisymmetric) parts, the relations between the time averages are given by

$$\overline{T(\psi, \dot{\psi})} = g_0 + \overline{g_1 x_1} + \overline{g_2 x_2} + \int_{-\infty}^{+\infty} w_{11}(\tau)\, \mathrm{d}\tau + \int_{-\infty}^{+\infty} w_{22}(\tau)\, \Phi_{22}(\tau)\, \mathrm{d}\tau + \int_{-\infty}^{+\infty} w_{12}^{(e)}(\tau)\, \Phi_{12}^{(e)}(\tau)\, \mathrm{d}\tau \tag{21}$$

and

$$\overline{\varrho(\psi, \dot{\psi})} = \int_{-\infty}^{+\infty} w_{12}^{(o)}(\tau)\, \Phi_{12}^{(o)}(\tau)\, \mathrm{d}\tau\,, \tag{22}$$

the indices e and o referring to "even" (symmetric) and "odd" (antisymmetric), respectively.

First, I would like to discuss Equation (22), which describes the "classical" optomotor reaction. Optomotor reactions in insects have been studied in great detail for many years (Hassenstein, Reichardt, 1956; Reichardt, Varjú, 1959; Varjú, 1969; Reichardt, 1961, 1969; Götz, 1972; Eckert, 1973; Buchner, 1974; Pick, 1974a, 1974b). The experimental results and their theoretical interpretation have led to the formulation of a two-receptor correlation model. Clearly the interaction between pairs of inputs must be nonlinear since the average output of every linear interaction depends only upon the mean values of the inputs and not on their relative phase relations. From Equation (22), one understands that the assumption of second-order nonlinear interactions leads necessarily (for the averaged output) to the cross-correlation of sensory data received by two receptor inputs. Hence if the time average of the reaction is considered, the correlation model is the most general representation of this type of motion detection. One of the strong experimental supports for second-order interactions arises from the property of so-called phase invariance: the time average of the optomotor reaction does not depend upon the relative phases of the spatial Fourier components of a pattern that is moved with constant velocity across the compound eyes. Every system describable with the Volterra formalism has this property if its order of nonlinearity is bounded by "two". For systems with nonlinearities higher than the second order, the property of phase invariance is strongly limited by specific

pattern conditions. We may conclude that the general property of phase invariance contains the necessary and sufficient requirements for nonlinear interactions not higher than the order of "two".

As has been mentioned before, the experimental determination of $\rho(\psi, \dot{\psi})$ carried out with a single black stripe environment has led to the result that ρ does not depend upon the position ψ (see Fig. 4). In spite of the ψ independence, it is doubtful whether the mechanisms responsible for motion perception are homogeneously distributed in ψ-direction, as the density of light receptor optical axes varies with ψ. The topology of the optomotor reaction mechanism has so far been disclosed only in part; it has been shown that the responsible interactions are not bound to next neighbor receptor inputs (Kirschfeld, 1972; Buchner, 1974).

The interpretation of Equation (21), which relates the direction-insensitive reaction part $T(\psi, \dot{\psi})$ to the corresponding even terms of the Volterra formalism up to the second order, is by far more complicated and not yet complete. It has been shown that stationary retinal images do not significantly contribute to the reaction behaviour; hence terms of zero and first order in Equation (21) have to be set to zero ($g_0 = \bar{g}_1 = \bar{g}_2 \equiv 0$). Consequently, the dependence of $T(\psi, \dot{\psi})$ upon the position ψ has, in principle, to be based on the contributions of the self- and the even cross-interaction terms. The experimentally determined $D(\psi)$ (Figs 3 and 4) can also be generated by flicker light, emerging from a small stripe source, mounted in different ψ positions (Pick, 1974a). The reaction to flicker disappears when the flickering stripe is wide (Pick, 1974b; see also Geiger and Poggio, 1975).

These results are in qualitative agreement with the hypothesis that the evaluation of position information from small objects is due to the contributions of the self-interaction terms in Equation (21), whereas the inhibitory influence mediated by the cross-interaction becomes critical only at larger receptor-to-receptor distances. In this connection, the result of the two-stripe experiment is important, as it proves the existence of mutual inhibitory influences for not too large an angular stripe separation.

More recent experiments have shown that the quantitative properties of even second-order cross-interactions fail to explain these new observations. Therefore Equation (21) has to be extended to higher order terms. These experiments are described here in some detail: test flies orientate towards a small black object (stripe segment) in front of a noiselike, contrasted background if the object undergoes at least small movements relative to the background. This observation was made under closed-loop conditions (Virsik, 1974). The effect can also be demonstrated when the foreground and the background are moved relative to the fly's eyes, and when the fixed flying fly is not coupled to its environment (open-loop conditions). The time average of the fly's torque reveals that the fly is not attracted by the small object if the object and the background are oscillating together coherently, with the same amplitudes. However when there is relative motion, the object is detected by the fly, which tries to turn towards it.

In order to quantify and characterize the detection effect, a series of open-loop experiments have been carried out where a black, vertically oriented stripe

(foreground) was sinusoidally oscillated around a given ψ position with a small amplitude ($\pm 5°$) and a fixed frequency (Heimburger et al., 1975). A 360° background panorama, consisting of randomly distributed contrast elements, was oscillated with the same amplitude and the same frequency in different phases relative to the stripe. The result of the experiment is given in Fig. 9. It shows that the fly does not "detect" the stripe when the phase relation amounts either to 0° (in phase) or to 180° (antiphase). The effect reaches its maximum for a 90°

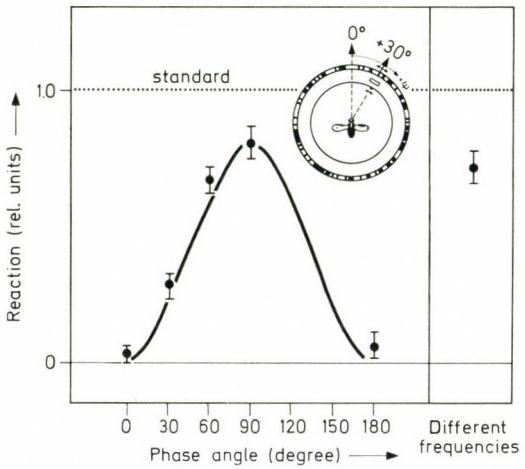

Fig. 9. Average torque responses (reaction from 55 experiments) plotted as a function of the phase angle between sinusoidally moving foreground and background. The frequency amounted to 2.5 Hz and the amplitudes to $\pm$ 5°. In the case of different frequencies, the foreground was moved with 1.8 Hz and the background with 2.5 Hz. Average positions of the stripe were $\pm 30°$. The average torque indicated in the figure by the filled circles was derived from the difference of the average reactions at the stripe positions $\pm 30°$. The vertical lines designate the standard deviations of the mean. The strength of the reaction was measured in units of a standard reaction elicited by the oscillating stripe only. The inset shows a cross section of the experimental setup. The dot on the inner circle indicates the stripe; the oblong between the inner circle and the outer one (random pattern) is a white non-transparent screen

phase shift. If the stripe and the background are oscillated with different frequencies, the effect is also strong. About half of the individual experiments included in Figure 9 were performed with a stationary opaque white screen interposed between the stripe and the background. No difference in the reaction has been found under these two different experimental conditions, suggesting the existence of lateral interactions between receptors that are stimulated by the oscillating stripe and receptors that receive stimuli from either side of the pattern and beyond the border of the screen.

Another important hint came from experiments (Pick, 1974b) employing two flickering light sources with defined phase relations; these experiments were the first to suggest the existence of lateral inhibitory interactions of order four

or higher. The phase dependence of the effect presented in Figure 9 is periodic in π and therefore can be fully accounted for by fourth-order nonlinear interactions between the signals received from two to four receptor inputs. It should be mentioned that a periodicity in 2π could be expected from the existence of second-order nonlinear cross-interactions. Hence these observations are in flat contradiction with the hypothesis that the only significant cross-interactions that exist are second-order symmetric ones. In Figure 10, two different types of fourth-

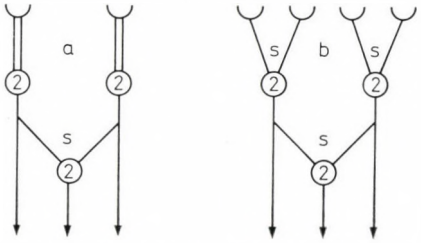

Fig. 10. Schematic representation of two- possible fourth-order interaction schemes built from graphs of second-order symmetric (even) cross- and self-interactions. (a) Two-input model; (b) four-input model. For details, see text

order interactions receiving their inputs either from two or from four receptors are given in terms of a two-step Volterra hierarchy. Each of the two steps involves second-order nonlinear interactions. The experimental results do not yet allow to differentiate between the two- and the four-receptor input models.

Let us now discuss the functional operations of the two models in a foreground-background experiment employing different phase relations. If the stripe is oscillated with a given frequency and small amplitude in front of one of the two receptor inputs (Fig. 10a) or one of the double receptor inputs (*b*), it generates a periodic signal of double frequency at the output of the second-order self- (a) or the second-order cross- (b) interaction level. In principle, the same holds true both in (a) and (b) for the outputs of the other branches that process the information received from the oscillating background. The frequency doubling by the upper level of the two models is of course accompanied by the doubling of the phase angle between the signals provided by the upper level interactions. Consequently, 0°, 90° and 180° phase shifts in the object-pattern motions are mapped into phase shifts of 0°, 180° and 360° at the output of the upper level. The second processing step on both (a) and (b) consists of a second-order cross-interaction that essentially evaluates the phase relations of the signals provided by the upper level interactions. Since 0° and 180° phase shifts in the object pattern space maps into 0° and 360° shifts at the output of the upper level, the outputs of the second-step cross-interactions are of equal sign, whereas a 90° phase shift, leading to 180° phase shift at the second level, results in the opposite sign. If one assumes that the output of the second stage cross-interaction is inhibitory

(negative sign), whereas the output of the upper level interaction is excitatory, one arrives at the qualitative behaviour presented in Figure 9.

An additional test of the fourth-order interaction scheme was carried out by changing the oscillation amplitude of the background, keeping all other parameters the same, for 0° phase shift between foreground and background. The result of the experiment is given in Fig. 11. The detection effect is strong for a zero relative amplitude of background; that is to say, the stripe oscillates and the back-

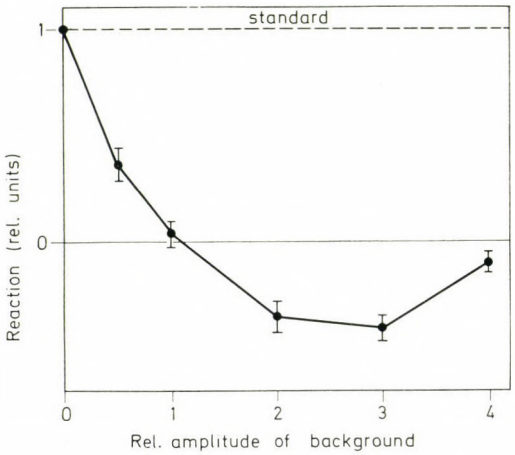

Fig. 11. Experimental details as described in the legend to Figure 9, except for different background oscillation amplitudes. The vertical lines designate the standard deviations of the mean

ground is at rest. For increasing background amplitudes, the inhibition increases, and the attractiveness effect reduces to zero for equal amplitudes of stripe and background (relative amplitude of background $= 1$). When the amplitude of the background is further increased, the inhibition overrides the excitation elicited by the stripe, and the detection effect changes sign. Under these conditions, the fly is no longer attracted, but instead repelled, by the oscillating stripe. Dynamic saturation at the motor output level is likely to affect these data (Figs 9 and 11). However, control experiments have shown that the qualitative properties of the effect (no object detection for 0° and 180° phase shifts; detection for 90° phase shift and for different frequencies) do not depend upon motor output saturation.

The foreground-background experiments, reported so far, have been undertaken with a 360° background panorama, consisting of randomly distributed contrast elements. More recently we investigated the properties of the symmetric lateral interactions with partially patterned backgrounds of the same class. The experimental results, obtained so far, suggest that the interaction range extends horizontally over the entire visual fields of both eyes. These results are not necessarily in conflict with the observations made with two vertically oriented stripes where it was found that mutual interactions disappear for angular displacements

of more than 80 degrees. They rather suggest that in case of a background pattern, long range interactions are mediated by the influence of pattern elements located inside the interaction range. These observations may find their explanation by an interactive neuronal net with recurrent interconnections.

In summary: The lateral fourth-order inhibitions described here can play an important role in the fly's ability to discriminate and to perceive patterns. Thus, for example, a discrimination of the environment into figure and ground, one of the fundamental processes in perception, could be performed by these interactions.

Concluding Remarks

Experimental evidence and theoretical considerations suggest that the visual orientation behaviour of the fly can be quantitatively predicted from the corresponding "open-loop" reactions by a phenomenological theory. The part of the visual system responsible for these reactions operates like a parallel organized network with many receptor inputs, interacting in a nonlinear way. Three types of nonlinear functional interactions are required for the explanation of the behavioural responses. One of them underlies the direction-sensitive optomotor response, related to a speed-dependent term in the phenomenological equation. The other two are responsible for the direction-insensitive response, which mediates the "attractiveness" of a pattern and appearsin the p henomenological equation as a position-dependent response.

Second-order (antisymmetric) cross-interactions between pairs of receptors compose the part of the network responsible for the direction-sensitive responses – the movement- (speed-) dependent evaluation. The two-dimensional topology of these mechanisms has only partially been unravelled so far. These interactions are present in both the upper and lower parts of the eye and seem to be rather homogeneously distributed.

Self-interactions provide the position-dependent response for narrow objects. They are weighted according to their two-dimensional location through gradients of their density and/or of their synaptic properties. Self-interactions would be consistent with the superposition property; the "attractiveness" of a pattern would be the sum of the "attractivenesses" of its components. However, inhibitory fourth-order symmetric cross-interactions also affect, at least on a certain angular range (between 5° and 80°), the position-dependent response and therefore violate the superposition property. The topology of this type of interactions is not known in detail, except that they are confined to the lower parts of the compound eyes (Geiger, Poggio, 1975). The interplay between the inhibitory fourth-order and the (excitatory) selfinteraction is responsible for the foreground–background discrimination and possibly represents the basic functional principle underlying spontaneous pattern discrimination in flies.

One of the prime questions remaining concerns the organization of the actual neural network underlying the interaction mechanisms. Due to the parallel

organization of the neural network, one may expect a correspondingly small number of sequential neural steps is very small.

The complex visual orientation behaviour rests on the cooperativity of a few types of elementary, but essential nonlinear interactions which are mainly organized in parallel.

References

Buchner, E. (1974) Bewegungsperzeption in einem visuellen System mit gerastertem Eingang, Dissertation, Fachbereich Biologie, University Tübingen, Germany

Eckert, H. (1973) Kybernetik *14* 1

Geiger, C., Poggio, T. (1976) Biol. Cybernetics *17* 1

Götz, K. G. (1972) Bibl. Ophthalmol. *82* 251

Graham, B., Haken, H. (1971) Z. Physik *243* 289

Hassenstein, B., Reichardt, W. (1956) Z. Naturforsch. *11b* 513

Heimburger, L., Poggio, T., Reichardt, W. (1975) Biol. Cybernetics *21* 103

Kirschfeld, K. (1972) The visual system of Musca: Studies on optics, structure and function. In: Information Processing in the Visual Systems of Arthropods (ed. R. Wehner), pp. 61—74. Springer-Verlag Berlin, Heidelberg, New York

Land, M. F., Collett, T. S. (1974) J. Comp. Physiol. *89* 331

Marmarelis, P. Z., McCann, G. D. (1973) Kybernetik *12* 74

Pick, B. (1974a) Z. Naturforsch. *29c* 310

Pick, B. (1974b) Das stationäre Orientierungsverhalten der Fliege *Musca*. Dissertation, Fachbereich Biologie, University Tübingen, Germany

Poggio, T., Reichardt, W. (1973a) Kybernetik *12* 185

Poggio, T., Reichardt, W. (1973b) Kybernetik *13* 223

Reichardt, W. (1961) Autocorrelation, a principle for the evaluation of sensory information by the central nervous system. In: Sensory Communication (ed. W. A. Rosenblith), pp. 303—317. MIT Press, Cambridge, Mass.

Reichardt, W. (1969) Movement perception in insects. In: Rendiconti della Scuola Internazionale di Fisica "Enrico Fermi" Corso XL III, pp. 465—493. Academic Press, New York

Reichardt, W. (1973) Naturwissenschaften *60* 122

Reichardt, W. (1973) Musterinduzierte Flugorientierung. Naturwissenschaften *60*

Reichardt, W., Poggio, T. (1975) Biol. Cybernetics *18* 69

Reichardt, W., Varjú, D. (1959) Z. Naturforsch. *14b* 676

Skorokhod, A. V. (1965) Studies in the theory of random processes, Reading, Mass.: Addison-Wesley

Stratonovitch, R. L. (1968) Conditional Markov processes and application to the theory of optimal control. New York: American Elsevier Publishing Company.

Varjú, D. (1959) Z. Naturforsch. *14b* 724

Virsik, R. (1974) Verhaltens-Studie der visuellen Detektion und Fixierung bewegter Objekte durch die Fliege *Musca domestica*. Dissertation, Fachbereich Biologie, University Tübingen, Germany

Virsik, R., Reichardt, W. (1974) Naturwissenschaften *61* 132

Virsik, R., Reichardt, W. (1976) Biol. Cybernetics, (in press)

Wehrhahn, C. (1974) Verhaltensstudie zur musterinduzierten Höhenorientierung der Fliege *Musca domestica*. Dissertation, Fachbereich Biologie, University Tübingen, Germany.

Wehrhahn, C., Reichardt, W. (1975) Biol. Cybernetics *20* 37

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Mathematical Biophysics

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1. In agreement with the general aims of this conference, it should be tried here to form a basis for the discussion of present tendencies and further orientation towards a special direction of biophysics. In contrast to the other presentations given here, the topic of this paper is hard to define and without doubt overlaps others, especially those of bioregulation, thermodynamics, kinetics, etc. This should not be of any account because problems of delimitation and definition are not of interest here.

In view of statements to be made later on, it should be pointed out here that, in my opinion, the general value of all biophysical research, that of mathematical biophysics included, is based on its ability to answer the special or general questions of biology. This has to be underlined because there is also the standpoint that the aim of the mathematical approach to biophysical problems may be arrived at by enriching physical or mathematical knowledge only. At least, this paper is meant to be only an incomplete attempt or a provocative basis for discussion.

2. The general tasks of mathematical biophysics are set by the particular role played by biophysics itself in the ensemble of biological disciplines. In contrast to biochemistry, the field of biophysics is not bound to a special level of biological organizational hierarchy at all. Moreover, biophysics has to concentrate on the problems of integrating these structural levels. This touches a central problem of modern biological investigation. The great achievements of molecular biology in the last few years have to be transformed and integrated into biological results of a general type. The behaviour of a system, however, is not due to the sum of its elements. Here, special types of interactions and feedbacks have to be allowed for.

These are problems necessarily to be solved chiefly by biophysics and especially by mathematical biophysics. In particular, the following tasks have to be formulated:

- Integration of sets of functional elements
- Investigations of correlations between structure and function
- Discovery of generalized principles

3. Without attempting to define mathematical biophysics, it has to be made clear what is here meant by this term. By way of elimination, it should be noted that questions of biometrics or mathematical analysis of special physical methods will not be discussed here. In general, the application of mathematics to solve biological problems is possible in two ways: on the one hand directly, and mediated by theoretical physics on the other. Strictly speaking, only the latter way can be called "mathematical biophysics". It is not possible, however, to draw a borderline between other combinations of the nouns "biology" and "biophysics" with the adjectives "mathematical" and "theoretical". We have listed 15 titles of published monographs and series containing all combinations possible (Table 1). This list is incomplete and, therefore, does not allow any significant differentiations. However, it shows two aspects:

Table 1

	Biology	Biophysics
Mathematical	3, 5, 8, 9, 11, 13, 15	4, 6, 7
Theoretical	2, 8, 12, 14, 15	1, 10

1. Beier, W.: Einführung in die theoretische Biophysik. Stuttgart, 1965
2. Bertalanffy, L. V.: Theoretische Biologie, Bern 1951
3. Fomin, S. V., Berkinblit, M. B.: Matematicheskie problemy v biologii, Moskva, 1973
4. Householder, A. S., Landahl, H. D.: Mathematical biophysics of central nervous system, San Antonio 1945
5. Lotka, A. J.: Elements of mathematical biology, New York 1956
6. Rashevsky, N.: Mathematical biophysics, New York 1938
7. Romanovsky, J. M., Stepanova, N. V., Chernavsky, D. S.: Chto takoe matematicheskaya biofizika? Moskva 1971
8. Waterman, T. H., Morowitz, H. J.: Theoretical and mathematical biology, New York 1965
9. Bull. mathemat. biology
10. Fortschritte der experimentellen und theoretischen Biophysik
11. J. mathematical biology
12. J. theoretical biology
13. Matematicheskie problemy v biologii, Moskva 1966
14. Na puti k teoreticheskoi biologii, Moskva 1970
15. Teoreticheskaia i matematicheskaia biologia, Moskva 1968

1. The combinations most frequently used are: "mathematical biology" (7 times) and "theoretical biology" (5 times) in contrast to "mathematical biophysics" (3 times) and "theoretical biophysics" (twice).

2. No significant differences of topics could be found in the papers contained by these books.

The reason for aspect No. 1 seems to be the following. The attribution of the given adjectives to the noun "biophysics" does not give any essential informa-

tion – biophysics without mathematics is impossible. The case of marking "biology" as "mathematical" or "theoretical" is quite different. Aspect No. 2 shows that differentiation between possible combinations of these four words is without any importance. The following question, however, is of general interest: Is "theoretical biology" identical with the other combinations listed here? This should be a point of further discussion and we will return to this question at the end of this paper.

4. What, in detail, are the actual tasks of mathematical biophysics? Based on the remarks above, a systematic survey of these tasks should be given for discussion. Special questions were tried to be summarized by comprehensive formulation (Table 2). Without being able to discuss all these topics in detail

Table 2

Mathematical biophysics

Mathematical direction

Calculation of metabolic sets
Cybernetic regulation of organ systems
Population kinetics
Neuronal networks
Models of growth and evolution

Theoretico-physical direction

Adaptation of basic physical equations to conditions due to special biological structures (e.g. Nernst-Planck eq., Poisson-Boltzmann eq., Navier-Stokes eq.)
Functional correlation of fundamental biophysical processes and properties (fluxes, potentials, charges, concentrations etc.)
Approaches of statistical mechanics for calculations of the problems of molecular biology
Quantum-mechanical calculations
Analysis of fluctuations
Biomechanical approaches

(some of them are treated in other papers read here), we will concentrate on general questions arising in this connection.

5. What is the applicability of these calculations to real biological problems and, in this context, what is the proportion of intensities in their investigations? It should be noted that the number of papers published in particular fields is obviously not dictated by biological necessity but frequently rather by the theoretical interests of physicists and mathematicians. This seems to be an important point for discussion.

This will be demonstrated by comparing the situation in mathematical modelling of the processes of nerve excitation with those concerning functional correlations within a fundamental biological system – e. g. the mitochondrion. Starting with the Hodgkin-Huxley equation, there is a gigantic framework of theoretical papers containing kinetic and cybernetic models and describing the action potential of the nerve in formal terms. A great number of them are l'art-pour-l'art plays without any biological significance.

In contrast, we have by now an immense mass of experimental facts for some relatively simple biological systems such as mitochondria or erythrocytes. This means results of measurements of fluxes, osmotic behaviour, changes of metabolic states etc. A theoretical connection of these facts is extremely necessary but recently there have been only first attempts in this direction.

The reason for this disproportion is evident: in order to construct models of the first-mentioned type, only a limited knowledge of experimental details and biophysical relations is necessary and this allows the application of experience in mathematics and cybernetics. In the later-mentioned case, a well-grounded knowledge of biological facts and biophysical statements is necessary and here calculations are possible only as a result of long experience and close contact with experimentalists. It is important to note that the rate-limiting phase in this process is not due to the recent state of development of thermodynamics!

6. Another question worth discussing here is the following: What type of mathematics do we need to solve these problems? What limitations arise from the mathematical point of view by integrating biological systems?

Increasing the complexity of calculated systems simultaneously increases the number of variables and, therefore, not only the difficulty of mathematical treatment and the extent of computer programmes, but also the ambiguity of the results obtained. Especially considering the natural variability of biological parameters, there is a propagation of errors producing a great uncertainty of results. On the other hand, regarding the biological system itself as an analogue computer for equations postulated (an unusual view, of course!), the actually observed confidence of its function indicates that there are correlations still unknown.

In recent time, some methods of topology have obtained increased importance. It seems worth focusing our attention on this development. The following question is open to discussion: Is it necessary to generally change the students' education in mathematics? The greatest advantage seems to be reached by applying network theories in cybernetics and thermodynamics.

7. Concluding these remarks, we repeat the question: Is mathematical biophysics able to represent theoretical biology? Firstly it should be pointed out that, in the future, mathematical biophysics has to fulfil an increasing work in solving the particular problem of metabolic or physiological networks. This is necessary not only for concentrating the information obtained by experiments but it has a great importance in medical diagnosis and therapy, regulation of plant growth and microbial production etc.

On the other hand, it seems that the framework developed by Thompson, Bertalanffy, Lotka, Rashewsky and many others has to be completed. We should look on this development with due modesty and in full awareness of the complexity of the biological system. The question whether theories of the living system can be completely formulated in mathematical terms depends chiefly on the development of mathematics itself. Understanding mathematics as the highest form of logical reasoning, all scientific knowledge should be formulable in its terms.

These prospectives assign to mathematical biophysics a responsible and important role in the ensemble of biological disciplines. This task is to be complemented by its applicability in the control and regulation of processes of life in Public Health, Agriculture and Industry.

Discussion

B. Pullman: It is necessary to make a difference between mathematical biology and theoretical biology. This latter consists of the fundamental concepts which form the basis of the mathematical treatment. This does not mean they are on different levels of importance.

I. C. P. Smith: Congruent mathematical tools are needed to solve the problems of biology; this is other than to apply existing mathematics to simple problems. A hard problem is the communication factor because the results of mathematical biophysics are ignored by biologists many time because they do not communicate very well.

A. Kotyk: We have to cope with the task of translating the mathematical language to the biologists. Perhaps proper textbooks of mathematics and theoretical physics for biologists are needed.

A. Ehrenberg: The mathematics is like a tool in the same way as a complicated physical instrument and to describe the results of biology we do not need to understand all the details, but we have to know what type of results we can get by applying mathematics.

B. Pullman: Biologists must understand mathematics as tools taking into consideration its limits.

P. O. P. Ts'o: The mathematics could be exceedingly powerful to integrate observation and understanding.

R. Glaser: It is impossible to educate a specialist who is a good physicist and a good biologist at the same time; we need the translation between pure physics and biology.

V. Vasilescu: It is necessary to develop new branches of mathematics.

B. Pullman: If the level of biological researchwork makes it necessary; perhaps we are not in this stage.

L. A. Blumenfeld: We have to teach biology to physicist if we want to produce a new type of scientist because as a rule it is impossible to teach physics to biologists in a necessary degree; but (in *J. Tigyi*'s opinion) it is a biologist – like Szent-Györgyi is – who is not only able to translate between the two main discipline but really synthetize their basic facts, functions and laws.

J. Jaz: Perhaps it is the best to collect the different people: biologists, mathematicians, physicists, chemists etc. and to create interdisciplinary ensembles. Another problem is the teaching of sciences in developing countries. Perhaps we have to write some books for them and UNESCO could do something in this direction.

B. Pullman: The IUPAB has a program to produce such books.

J. Tigyi: The program of the Hung. Acad. Sci. to develop the integrated teaching of natural sciences can be a contribution to the solvation of this problem.

I. C. P. Smith and *A. Goldbeter* has the opinion that well organized groups and teams are more useful to perform successful researchwork in biophysics.

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Biophysics of Muscle Contraction

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1. The importance of muscle research is generally appreciated because of the special role of muscle function of all animal living organisms. There is a very remarkable estimation of Kapitsa – made 20 years ago–that the major part of the total mechanical energy produced all over the world is generated by muscle machines.

It is also well known, that two thirds of the human body consists of muscle tissue and a significant part of the metabolism goes on even in the resting muscle. This participation becomes the predominant component of the total metabolism during physical work or sport exercises.

But the muscle machine has its importance not only from biological point of view but also from technical one. The bionics has long tried to imitate the muscle because of its high mechanical efficiency, isotermic operation, noiseless and extremely reliable action.

2. Corresponding to these facts muscle research has a very great tradition, and the elaboration of exact methodology of investigation of muscular activity started in the last century. Du Bois Raymond, A. Fick, H. Helmholtz contributed with very basic scientific statements to this work. If we study the history of muscle research we can observe that all the modern methods of physics, chemistry and physical chemistry have been applied and utilized very soon, and there are cases in which the physical method has been developed or perfected on biological objects and by biologists.

The muscle biophysics is a significant part of the biophysics. There are many famous centers of muscle biophysics in China, Japan, UK, USA and USSR. In Hungary about 15 per cent of biophysicists have close relation with muscle research.

3. The present state of the muscle research. It is not easy to take in the situation because of the tremendous amount of experimental data, which are very often inconsistent with each other, gained on different experimental objects, under different conditions. So the theoreticians can find experimental evidences for all kind of theory.

Hovewer, if we want to give the classification of the experimental facts about muscles the best way is to do this according to the phenomena of the succes-

sive stages of the muscular activity. There are four more or less separate groups of the phenomena; these are as follows:

a) Initial phase, excitation
b) Excitation-contraction coupling
c) Contraction
d) Tension development.

a) In the case of striated frog muscle, the initial phase requires 2 – 5 ms (at room temperature), therefore the most valuable are those experimental methods which have proper high time resolution and sensitivity.

The electric phenomena are the most important characteristics of this phase. It is evident that the largest part of the research work tries to understand these events. The *membrane theory* of the excitation of nerves worked out by Hodgkin and Huxley has been applied to the muscle excitation, too. The basis of this theory is the reversible change of the permeability of muscle membrane by depolarization. The energy of the excitatory processes would be supplied by the concentration gradient of the ions localized in different concentration on both sides of the membrane and forming a concentration battery. This very elegant and attractive theory is useful, the only trouble is that it is not true, and it is unable to explain a lot of experimental facts.

We do not want to go into details, we want to present only our most important arguments.

The basis of the membrane theory is the exclusive role of the ions in the excitation of muscle. This opinion supposes that all the ions behave like free diffusible ions in a real solution and does not take into consideration the facts that:

1. The muscle is a colloid system, containing protein in amount of its 1/5 volume. Under these circumstances a certain part of the muscle water is bound to the protein molecules, the connection of ions present are also not uninfluenced by the protein and many microwave measurements prove a bound state of about 2/3 of potassium in muscle (Masszi, Tigyi-Sebes, 1962). The radioautographs made by ELMI show a nonuniform distribution of potassium (Kállai, 1969) etc. (Fig. 1).

2. There are lots of evidences that the muscle tissue has to be considered as a quasicrystalline system (see April's liquid-crystalline concept; 1975), where the electronic charge transfer is an important factor in the generation of bioelectric phenomena. The data of the electric conductivity of muscle membrane, its temperature coefficient (Nagy, 1970) (Fig. 2), the role of trace elements in the electrical conductivity, the thermocurrent, the photodynamic properties suggested us to treat the muscle with the concept of the solid state physics. A hypothesis has been worked out in our institute during the last two decades, according to this we consider the muscle tissue as an electric semiconductor system (Ernst, 1956; 1963). A lot of experimental fact of muscle excitation seems to be consistent with this hypothesis.

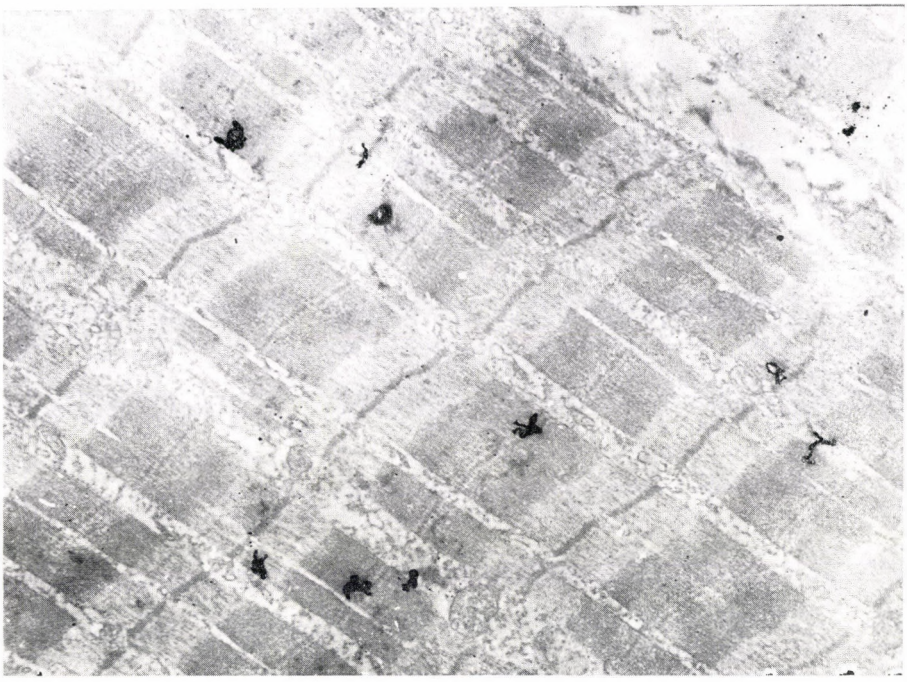

Fig. 1. ELMI radioautogramm. The localization of K^{42}-atoms in the cross-striated muscle

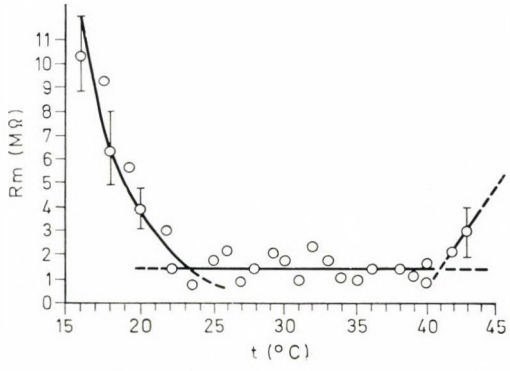

Fig. 2. The temperature dependence of the electric resistance of the cross-striated muscle-membrane. (Nagy, L. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 341)

Other important phenomena of the excitatory phase are:
latency relaxation

volume decrease

optical changes { transparency
spectra in grating
absorption spectra

These data become more and more reliable and exact, parallel with the methodological development of physical and technical instrumentation.

The most promising experiments are those which try to find relation between these phenomena gained by different method. E.g. the volume decrease is a sign of conformational change and it shows a very close synchronism with the action potential (Ernst et al., 1951; 1954) (Figs 3 and 4).

b) Excitation-contraction coupling.

There are many well-known methods which can stop the propagation of the excitation to the contraction, e.g. hypertonic solution (Varga-Mányi, Tigyi, 1962), or isotonic solution in heavy water can separate the excitation from the contraction in a reversible way. The Ca^{++} ions supposed to serve as triggers of the muscle contraction but many obscure problem has to be solved before we could give a complete theory of these events.

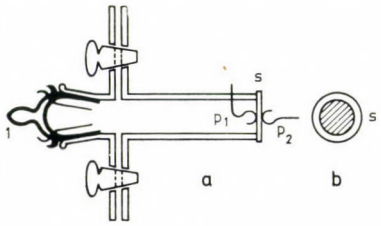

Fig. 3. The piesoelectric volume-meter for the detection of volume changes of the muscle during contraction (Ernst, E., Tigyi, J., Örkényi I. (1951) Acta Physiol. Acad. Sci. Hung. *2* 281)

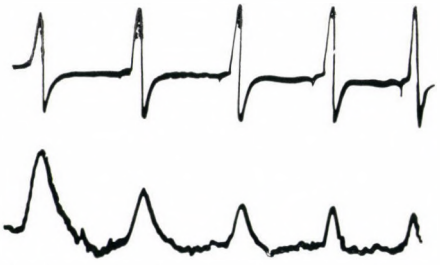

Fig. 4. The action potential- (above) and volume decrease — (below) curve in a tetanic contraction. (Ernst, E., Tigyi, J., László, M. (1954) Acta Physiol. Acad. Sci. Hung. *6* 171)

c) Based on X-ray studies, it is supposed that the muscle proteins do not show molecular structural change during contraction. The cross-striations – studied by ELMI – could show such pictures which suggested the ideas of the *sliding filament* hypothesis by H. E. Huxley (Hanson, Huxley, 1955). This is a widely accepted model of muscular contraction but there are strong critical arguments based on experimental facts, which make the validity of this hypothesis very problematic:

The elements of this hypothesis: the thick and thin filaments are not well defined from physicochemical point of view. The cross-bridges – which would serve for driving (or sliding) force of the sliding of the filaments – have theoretically very peculiar molecular mechanisms.

There are many experimental facts which show that the thick filaments are not unchanged, they can vary their length and their diameter, too (Frank, Strankfeld, 1973).

There are some sorts of muscular contraction in which the classic cross-striation is absent (e.g. that of smooth muscles) still the contraction comes about. Time resolved X-ray studies are under development, and we hope it will give a more exact explanation of molecular changes and mechanism of muscle shortening.

d) The tension development.

If the contracting muscle is hindered from shortening it develops a very great tension (at mammalian 10 kp/cm^2). The tensionless contraction differs principally from that one with tension. The development of the active and passive tension evokes a significant change in the structure of the muscles. An increasing stiffness indicates that a profound conformational change takes place because of the tension. The complex investigation of mechanical phenomena, heat produc-

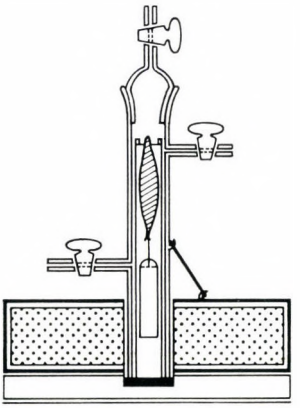

Fig. 5. The "magnetic" volume-meter for measuring the volume changes in the muscle under active and passive tension. (Ernst, E., Tigyi, J. (1951) Acta Physiol. Acad. Sci. Hung. *2* 243)

tion and optical changes suggested us to work out a hypothesis of the polymer crystallization of the muscle proteins caused by stretch (Ernst, 1963; Ernst, Tigyi, 1951; Tigyi, 1959; Lőrinczi, 1974) (Figs 5 and 6). According to this opinion a regular steric molecular arrangement will be produced automatically by the mechanical tension. The extra "crystallization-heat", the increase of birefrigence, the volume changes etc. support our hypothesis very consistently. The synchronous

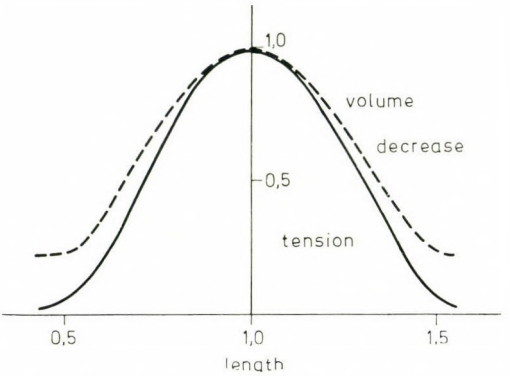

Fig. 6. The active tension and the volume decrease of the muscle as the function of the muscle length

measurement of the heat and conformational changes would give further strong evidences of this idea. The synthesis of the results of muscle protein biochemistry is indispensable, X-ray studies would also be necessary for completing this hypothesis.

4. The theories of muscle contraction

As I mentioned, the wide variety of diverse experimental data give good opportunity to the theoreticians for working out theories. All the theories of contraction all have a common deficiency. All they are extremely one-sided.

The theory of muscle contraction has to include the explanation all the mechanical-chemical structural and energetical phenomena; e.g. the Hill-Meyerhof theory have considered the chemical and energy changes, the ATP theory worked out by Engelhardt, Szent-Györgyi, etc. have considered the mechanical and chemical changes, the sliding filament theory was based only on the structural and mechanical changes and all they neglected other main aspects of the whole muscle problem.

The muscle research has now the main task to develop a complex theory of muscular activity based on the most modern experimental results gained by highly sensitive, time-resolving, exact methods of physics, chemistry, solid-state physics, X-ray, UV, visible, and IR spectroscopy. The synthesis of these

results under the control of quantum biochemistry can lead us to form a real complex theory of muscle contraction.

This work needs a well organized strategy for the managing of muscle research. The final goal of muscle research will be the construction of an artificial muscle machine, but we have to be very satisfied during the next decade, if we will be able to find new exact – always reproducible – experimental facts, which can help us better understand the physiological and pathological phenomena of muscle.

References

April, E. W. (1975) Nature *257* 139
Эрнст, Й. (1956) Биофизика *1* 296.
Ernst, E. (1963) Biophysics of the Striated Muscle, Akadémiai Kiadó, Budapest
Ernst, E., Tigyi, J. (1951) Acta Physiol. Acad. Sci. Hung. *2* 243
Ernst, E., Tigyi, J., Örkényi, J. (1951) Acta Physiol. Acad. Sci. Hung. *2* 281
Ernst, E., Tigyi, J., László, M. (1954) Acta Physiol. Acad. Sci. Hung. *6* 171
Frank, G., Strankfeld, J. (1973) Moderne Medizin *3* 10
Hanson, J., Huxley, H. (1955) Symp. Soc. Exper. Biol. *9* 228
Kállay M. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 71
Lőrinczi, D. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 383
Masszi, G., Tigyi-Sebes, A. (1962) Acta Physiol. Acad. Sci. Hung. *22* 273
Nagy, L. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 341
Tigyi, J. (1959) Acta Physiol. Acad. Sci. Hung. *16* 129
Varga-Mányi, P., Tigyi, J. (1962) Acta Physiol. Acad. Sci. Hung. *22* 287

Discussion

L. A. Blumenfeld expressed his doubts about the power of the experimental facts introduced by the lecturer as proves of the semiconductivity of excitable tissues.

J. Tigyi mentioned other proves: the sensitivity of muscle against trace elements, the photodynamic effect.

L. A. Blumenfeld: What are the charge carriers?

J. Tigyi: In artificial membranes electronic conduction surely exists; charge transfer complexes, holes, excitons may play a role. Do not forget the muscle has a quasicrystalline structure.

B. Pullman gives an outline history of the semiconductor investigations in biology. He thinks the most important is to look for the origin of the semiconductivity, to find out the nature of the charge carriers involved in order to solve a typical biophysical problem.

J. Tigyi gives some more details of the semiconductor story, namely about Calvin's work. He accentuated the need of an extensive collaboration in this field.

C. Helene: The mechanism of vision, too, can be connected with this problem.

I. C. P. Smith: We need a synthesis of the role of proteins and of the role of membranes which have a conformational change during the excitation.

J. Tigyi: It is not too easy to define the muscle fiber membrane. Experiments of Podolsky, Natory and Garamvölgyi show that no membrane is needed to the muscle contraction. The surface has an important role, but it is not the scene of all the events assumed by Hodgkin and Huxley.

S. Maričić: thinks that NMR could give some more informations, if one used D_2O.

I. C. P. Smith has some doubts about the reliability of experiments performed with D_2O.

P. O. P. Ts'o and *J. Tigyi* stress that actin has an important role, its conformational changes are responsible for all sort of contraction in the animal world.

B. Pullman: proposes an international collaboration in the investigation of water structure around protein molecules, the water may be bound to them.

R. Glaser: The membrane is the control system of contraction; the muscle biophysics involve membrane and protein biophysics.

C. Helene: The movement of protein molecules in the membrane can be interesting, too.

O. Kratky: The membrane proteins may be investigated by small angle scattering.

J. Tigyi: The crystallization of muscle tissue during active or passive tension makes us think that the sliding filament hypothesis is dead, if we want to describe the muscle contraction we have to use the terms of polymer chemistry. To sum up: there are 4 main directions of collaboration: 1. Biological semiconductivity, 2. Water binding, 2. The general problem of biological movement; intracellular movement involved, 4. High time-resolving X-ray investigations.

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Membranes and Transport

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The recent rate of growth of research interest in biological membranes has few parallels in the history of science. Membranes are studied by mathematicians, physicists, chemists and biologists in a world-wide effort to harmonize their multifarious functions with their fundamentally simple overall structure. The ubiquitous occurrence of membranes in biological objects attests to the usefulness of this particular structure both for survival and for smooth running of various processes in living cells. Of the many functions of biological membranes, ranging from providing mechanical support to cells (in protozoans and Mycoplasma, for example) to acting as the locate of many enzymes (especially in mitochondria and bacterial membranes) to permitting or preventing the passage of solutes from one compartment to another, to receiving and transforming various chemical, electrical and optical signals, to functioning as a biological clock, the primary (evolution wise) function and one that is truly biophysical in nature, is that of transporting molecules and ions from one solution to another. This function is apparently two-fold: One of its aspects is to maintain a more or less constant intracellular milieu, particularly with respect to cations, the other has to do with translocation of nutrients into and of products (either waste or those with a mission) out of cells.

After years of dispute we are fortunate in possessing now a generally acceptable model of membrane structure, that of the fluid mosaic type. While it preserves a continuous lipid bilayer as the main structural feature it provides for functional flexibility in two ways (Fig. 1): By inserting some of the proteins into the lipid bilayer either isolated or in clusters or even in regularly organized arrays (rhodopsin or cytochrome oxidase, for instance) – these are the so-called integral or intrinsic proteins; by placing the rest of the proteins on the membrane surface, either in a random fashion (some of the periplasmic proteins in unicellular organisms) or in a relatively rigid framework (erythrocyte spectrin) – these are the so-called peripheral or extrinsic proteins.

Most of the latest results of analytical work and of probing into membrane structure with spin and fluorescence labels bear out the fluid mosaic model and it is now a major concern of the synthesis-minded membranologists to bring isolated components together in such a way as to simulate at least some of the native membrane functions.

It lies perhaps at the very core of progress in scientific research that the selection of experimental material follows a cyclic pattern. Macroscopic transport in whole animals, plants and organs was once studied by the early physiologists, to shift later to the cell level and finally to the level of membranes themselves as the required techniques were developed. Now that a great deal is known about membranes, the interest is back again on the integration of various membranes and their functions within the cell and there are signs of moving up to the whole organs again, with their sophisticated regulations, etc. (The same course was followed by the geneticists who started with higher plants to move to *Drosophila* chromosomes and finally to *Escherichia coli* and lambda bacteriophage with their individual molecules – now the trend is again back to the eukaryotic genome and multicellular organisms.)

The present-day biophysicists is still confronted with problems of transport

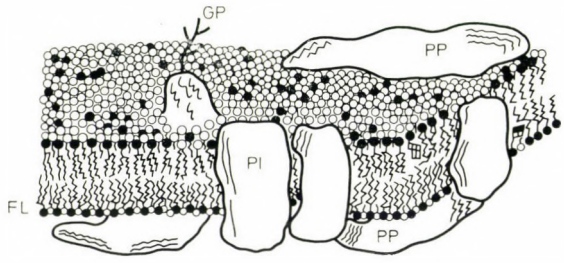

Fig. 1. A schematic view of the fluid mosaic membrane with FL the phospholipid bilayer, PP the peripheral proteins, PI the integral proteins and GP glycoproteins

at the membrane level (Fig. 2). Many nonelectrolytes and some ions can cross the membrane by a process characterized as simple diffusion although interactions with membrane components, either hydrophilic (moving through polar protein domains) or hydrophobic (moving through the lipid domains) no doubt take place. Evidence for this derives among other things from the high activation energy of these processes. Still, they are nonspecific and nonsaturable.

The majority of substances, however, penetrate into cells by processes involving a specific membrane protein and hence show a specificity similar to that of enzyme reactions and the process tends to saturation at high substrate concentrations. They may proceed without requirement for energy or may require a source of energy. Finally, transport systems where the transported solute is chemically modified have been described and are designated as group translocation. While the phenomenological descriptions of most transport processes are satisfactory (chemical kinetics and thermodynamics of irreversible processes having played a substantial role here) there are two major unresolved problems on the physical or molecular level. The first is the nature of coupling of transport processes with energy. According to a strict definition, active transport is directly coupled to an energy yielding chemical reaction – through an extension of the definition

it may also be coupled to a flow of another solute which, in its turn, has built up a difference of chemical or electrochemical potential by a truly active transport. While the flow-to-flow, or secondary, coupling can be envisaged in the form of a "carrier protein" binding both the driving (usually ionic) species and the driven (often nonelectrolyte) species in a ternary complex, the primary coupling is usually somewhat vague and frequently is concealed under the term "energized membrane". This energization may occur through ATP or polyphosphate splitting, through substrate oxidation via a redox system and possibly through interaction with energy in the form of electromagnetic radiation. The cases of Na, K-adenosine triphosphatase and of Ca-adenosine triphosphatase have been brought farthest in respect of molecular detail but even here the way energy obtained from ATP hydrolysis is used mechanically to move the molecule of the ion-binding subunit is far from clear.

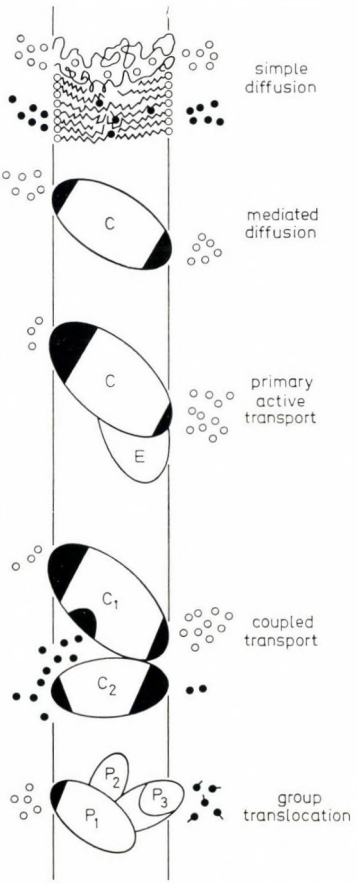

Fig. 2. Different types of membrane transport with C the "carrier", E. the source of energy and P group translocation protein

This brings us to the second major problem. The term carrier has been used both knowledgeably and not to describe an entity in the membrane which brings about the translocation of substrate (unless it passes by simple diffsion). On present evidence, saturable transport may possibly occur through the mediation

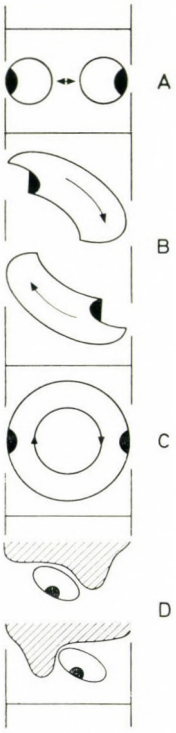

Fig. 3. Different modes of carrier movement in the membrane. A, translational diffusion; B, conformational flip-flopping; C, rotation; D, gate mechanism where the carrier itself does not move. Mechanisms B and D are more likely than A and C

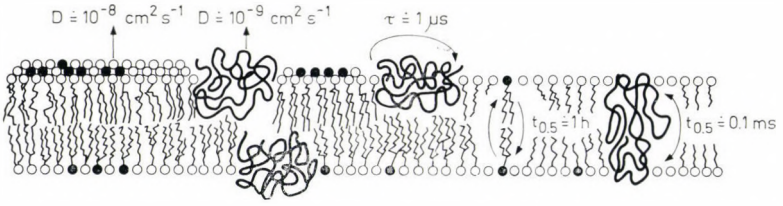

Fig. 4. Basic types of movement of membrane components. Lipids as well as proteins diffuse laterally with the diffusion coefficients shown. Some proteins (rhodopsin, bacteriorhodopsin) rotate in the monolayer or possible entire bilayer about an axis perpendicular to the membrane with relaxation times ranging from fractions of μs to ms. Phospholipids flip back and forth very slowly, with half-times of hours. Carrier proteins must perform this function much more rapidly, the value of 0.1 ms for the half-time having been computed for glucose transport in baker's yeast

of a single protein which changes its conformation so as to expose its binding site to the one or to the other membrane face (Fig. 3). If a change of affinity occurs simultaneously, active transport can ensue. On the other hand, as shown by genetic studies, there exist binding proteins in bacteria, yeasts and fungi that are only a part of the transport system. How this binding protein transmits the substrate to the carrier proper is unknown. It should be noted that more of the movements of membrane components investigated recently by physico-chemical methods (translational diffusion, rotation, flip-flop, Fig. 4) can account for the movement of the "carrier" across the membrane.

Appendix

To ensure progress in these and related fields two approaches may be recommended.

(1) To join forces with specialists in other fields, e. g. theoretical physics, and polymer chemistry.

(2) To exchange views whenever considered useful, with "related" scientists in other countries.

The principal obstacle in point one (leaving aside the question of finances and possibilities of selecting the right person) is in finding a common language that would make accessible the heights of modern thermodynamics to the protein chemist or the biologist (it is always more difficult than the other way around). Those capable and willing to do this "translation" are not many. However, the possibility of receiving specialized training in a well-equipped laboratory and/or under the guidance of a renowned expert should not be overlooked in this connection.

The second point should perhaps receive special attention. The exchange of views could be two-fold: (a) spontaneous, on the spur of the moment, when an urgent need arises; (b) planned, in the form of conferences, more or less regular. The first type still leaves much to be wished for in the way of actual operability. The second type is somewhat more easily accomplished and it is particularly useful (i) when the meeting is restricted in size by devoting it to a sufficiently narrow topic, and (ii) when it is truly international, bilateral meetings being of less value as a rule. The conferences may be organized in the form of summer or winter schools – the ones supported by FEBS or those under the auspices of the Polish Academy of Sciences were most laudable undertakings.

Although I do not wish to advocate a proliferation of committees and councils a certain level of mutual information among membranologists should be perhaps maintained in a more formal fashion than has been done so far. A body of specialists from various countries in Europe might be constituted to ensure this goal.

Discussion

R. Glaser; The investigation of transmembrane gradients is very important because they are connected not only with excitation but with many other phenomena.

C. Helene: One of the most significant point is to investigate the *information transfer* across the membranes; the other is the question of the role of protein molecules built in the membrane.

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Thermodynamic and Kinetic Aspects of Regulation

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Introduction

The extension of the Thermodynamics of irreversible processes to the nonlinear range has revealed that new types of phenomena can take place in chemical systems, beyond a critical distance from equilibrium (Prigogine, 1967; Glansdorff, Prigogine, 1971). Whereas near equilibrium the theorem of minimum entropy production at the stationary state ensures the stability of this state, far from equilibrium, stability is not more ensured. New types of coherent behaviour are observed beyond the point of instability of the nonequilibrium stationary state. This behavior consists in a spatiotemporal organization (sustained oscillations or chemical waves), or in all-or-none transitions between multiple steady states. These dissipative structures are maintained in open systems only, and a necessary prerequisite for their occurrence is nonlinearity of the kinetic equations governing the system in which they arise (Prigogine, 1967; Glansdorff, Prigogine, 1971; Nicolis, 1971).

It can be anticipated that the conditions for the occurrence of dissipative structures are frequently satisfied in biochemical systems. Indeed, the allosteric enzymes that control cell metabolism usually function far from equilibrium, and are subject to multiple types of regulation. The cooperativity of these enzymes and the existence of feedback processes render biochemical kinetic equations nonlinear. Furthermore, a living cell is the most typical example of an open chemical system. The above properties favor the occurrence of instabilities in enzymatic reactions beyond a critical distance from thermodynamic equilibrium.

The most frequently encountered type of dissipative structure consists in temporal organization. Several examples of oscillatory enzyme reactions have by now been demonstrated (Hess, Boiteux, 1971; Goldbeter, Caplan, 1976). We shall consider in turn glycolytic oscillations and the periodic synthesis of cyclic AMP. A theoretical analysis of allosteric models for these two oscillatory systems shows how coherent spatiotemporal behavior originates from cellular metabolism, and throws light on the molecular mechanism of instability in biochemical pathways.

Glycolytic oscillations

Glycolytic oscillations are the prototype of periodicity in a metabolic pathway (see ref. Hess, Boiteux, 1971; Goldbeter, Caplan, 1976 for a review). These oscillations, observed in single cells and cell populations of yeast, as well as in yeast and muscle extracts, result from the cooperative and regulatory properties of phosphofructokinase (PFK). Higgins (1964) and Sel'kov (1968) have proposed models for the oscillatory PFK reaction, based on the activation of the enzyme by a reaction product. These studies have been extended by consideration of an allosteric model for the PFK reaction (Goldbeter, Lefever, 1972; Goldbeter, Nicolis, 1976), in the frame of the concerted transition theory of Monod, Wyman and Changeux (1965). The model takes explicitly into account the cooperative properties of PFK; it represents an open K-V system in which the product is a positive effector of the dimer enzyme (Fig. 1).

The time evolution of metabolite concentrations in the allosteric model for PFK is governed by the following differential equation:

$$d\alpha/dt = \sigma_1 - \sigma_M \Phi$$

$$d\gamma/dt = \sigma_M \Phi - k_s \gamma$$

with

$$\Phi = \frac{\alpha(1+\alpha)(1+\gamma)^2 + L\Theta\alpha\, c(1+\alpha c)}{L(1+\alpha c)^2 + (1+\alpha)^2(1+\gamma)^2} \tag{1}$$

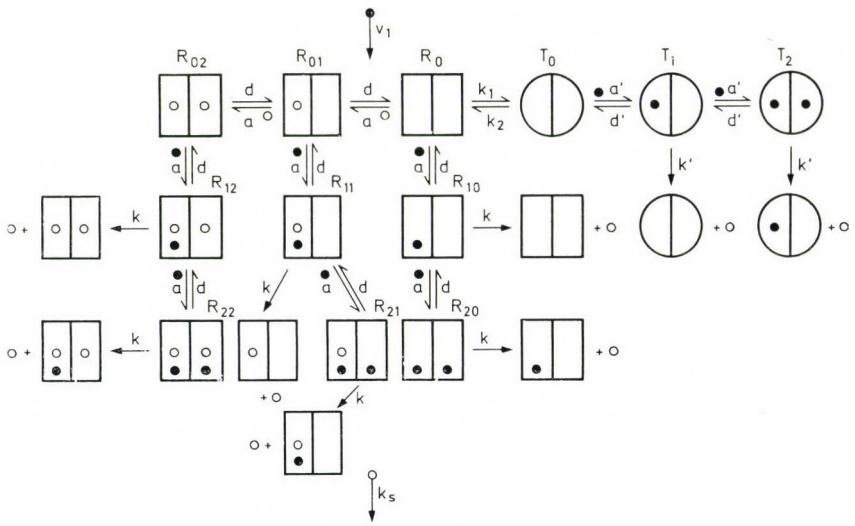

Fig. 1. Model of a dimer allosteric enzyme activated by the reaction product (Goldbeter, Lefever, 1972; Goldbeter, Nicolis, 1975). The enzyme exists under two conformations, *R* and *T*. The transition between these conformations is fully concerted (Monod et al., 1965). The *R* state has a larger affinity for the substrate ($a/d \geq a'/d'$) and a larger catalytic activity ($k \geq k'$) than the *T* state. The substrate (●) is supplied at a constant rate v_1, whereas the product (○) binds exclusively to the *R* conformation

where α and γ are the normalized concentrations of substrate (ATP) and product (ADP), respectively. Function Φ is identical with the ratio (v/V_M) for PFK. Further details on the definition of the model parameters are given in refs. Goldbeter, Lefever (1972) and Goldbeter, Nicolis (1976).

The stability analysis of the kinetic equations indicates that sustained oscillations of the limit cycle type can occur in this model, around a nonequilibrium unstable stationary state, as shown in Fig. 2 (Goldbeter, Lefever, 1972; Goldbeter, Nicolis, 1976). The system then evolves toward a periodic solution of unique amplitude and frequency, regardless of initial conditions. Glycolytic oscillations therefore represent a temporal dissipative structure.

The oscillatory behavior of the PFK model is compared in Table 1 with experimental findings in glycolyzing yeast extracts. Agreement is reached as to the oscillatory range of substrate injection rates, periodic substrate input phase shift by the product, entrainment by a periodic source of substrate, and stability in the presence of random variations of the substrate input (Boiteux et al. 1975). It follows that the oscillatory dynamics of the glycolytic system can satisfactorily be described by the allosteric model for PFK.

A detailed analysis of the molecular mechanism of instability in the PFK reaction shows that positive feedback and enzyme cooperativity play a primary role in the onset of periodic behavior (Goldbeter, Lefever, 1972). It should be noted that the model system operates at an infinite distance from thermodynamic equilibrium, as the substrate input, the catalytic step, and the product sink are taken as irreversible.

The results on oscillation in the PFK model (Table 1) have been obtained under the assumption of continuous strirring, as in experiments with yeast or muscle extracts. When the effect of diffusion ceases to be negligible, new types of dissipative structures can arise in the form of standing or propagating biochemical waves (Fig. 3). These spatiotemporal structures strongly depend on the geometry of the system and on the boundary conditions. Calculations with the PFK model show that the characteristic space scale of propagating waves is of the order of 0.1 cm (Goldbeter, Nicolis, 1976; Goldbeter, 1973). Experimentally, concentration waves are known to occur in the Belousov-Zhabotinsky reaction, which is the best-known example of oscillatory system in nonbiological chemistry (Zaikin, Zhabotinsky, 1970).

Periodic synthesis of cyclic AMP in the cellular slime mold D. discoideum

The life cycle of the slime mold *Dictyostelium discoideum* divides into a unicellular and a multicellular stage. When deprived of nutrients, amoebae of this species aggregate around centers by a chemotactic response to cyclic AMP (cAMP), and further develop into multicellular fruiting bodies (Bonner, 1971). The process of aggregation has a periodicity of several minutes. Indeed, waves of inward amoeboid movement appear to propagate outward from the center to the periphery of the aggregation field. This has long suggested that aggregation

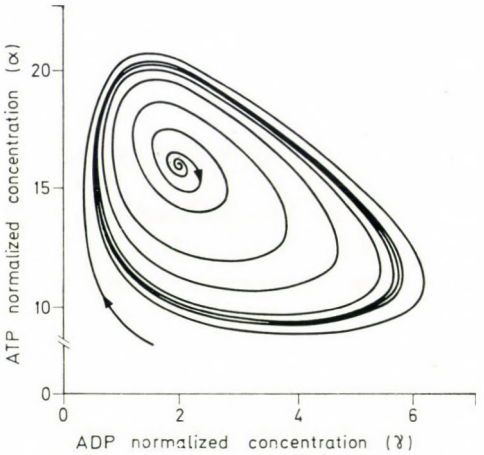

Fig. 2. Limit cycle in the allosteric model for PFK (Goldbeter, Lefever, 1972; Goldbeter, Nicolis, 1976). A unique closed curve is reached in the (α, γ) phase plane, irrespective of initial conditions. This solution corresponds to sustained temporal oscillations in the concentration α and γ

Table 1

Comparison of oscillatory behavior in glycolysis for a constant source of substrate (Boiteux et al., 1975)

Sustained oscillations	Model	Experiment
Oscillatory range of substrate injection rate (v_1)	19–246 mM/h	20–160 mM/h
Period	Of the order of min; decreased by a factor ≤ 10 as v_1 increases	Of the order of min; decreases by a factor ≤ 10 as v_1 increases
Amplitude	In the range $10^{-5}-10^{-3}$ M; passes through a maximum as v_1 increases	In the range $10^{-5}-10^{-3}$M, passes through a maximum as v_1 increases
Periodic change in PFK activity (in per cent V_M)	Minimum: 0.95; maximum: 73; mean: 17.5; activation factor: 77	Minimum: 1; maximum 80; mean: 16; activation factor: 80
Phase-shift by ADP	Delay of 1–2 min upon addition of 0.7 mM ADP (14 units of γ) around the minimum of ADP oscillations of 5 min period; small phase advance when the addition precedes ADP maximum	Delay of 1.5 min upon addition of 0.7 mM ADP at the minimum of ADP oscillations of 5 min period; small phase advance when the addition precedes ADP maximum

centers release periodically the chemotactic attractant; other cells respond to this signal by moving toward its source and by relaying the cAMP pulse (Shaffer, 1962; Gerisch, 1968).

Both the relay (Roos et al., 1975; Shaffer, 1975) and the oscillation (Gerisch, Hess, 1974; Gerisch, Wick, 1975; Gerisch et al., 1975) of cAMP have been dem-

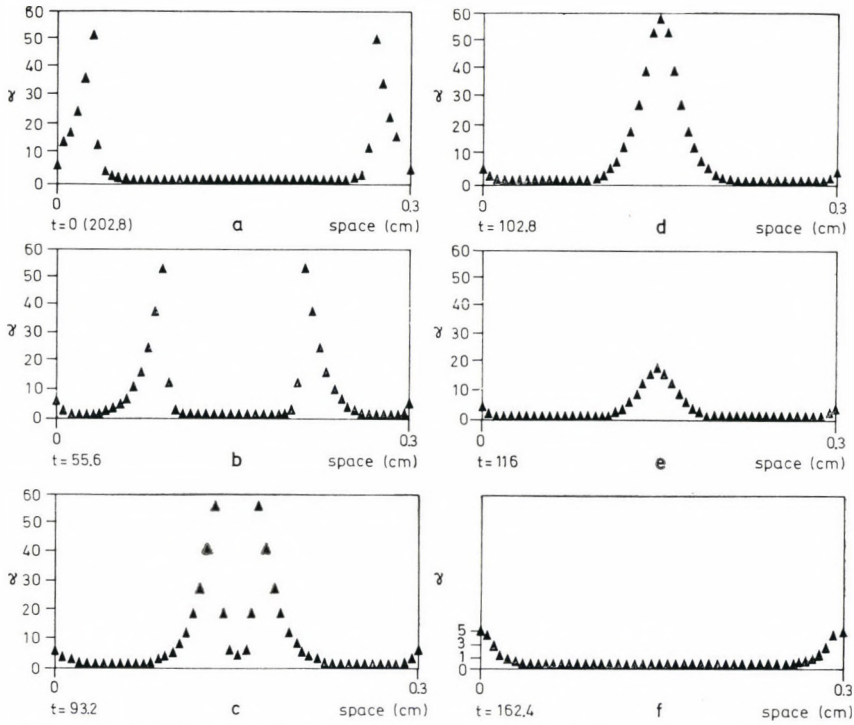

Fig. 3. Propagating biochemical wave in the PFK model Goldbeter, 1973; (Goldbeter, Nicolis, 1976). The spatial profile of the concentration γ of the reaction product is indicated at different time intervals over a period ($T = 202.8$ s)

onstrated experimentally in suspensions of *D. discoideum* cells. A model has been proposed for the oscillatory synthesis of cAMP (Goldbeter, 1975). The model is based on experimental data concerning the regulation of two enzymes involved in cAMP metabolism (see Fig. 4a), namely, adenyl cyclase and ATP pyrophosphohydrolase, which transform ATP into cAMP and 5'AMP respectively. According to Rossomando and Sussman (1973), in *D. discoideum*, adenyl cyclase is activated by 5'AMP whereas the pyrophosphohydrolase is activated by cAMP. Both enzymes are considered in the model as obeying the concerted allosteric mechanism (Monod et al., 1975). The time evolution of metabolite concentrations

is given by the kinetic equations (Goldbeter, 1975).

$$\begin{aligned} d\alpha/dt &= v_1 - \Phi_1 - \Phi_2 \\ d\beta/dt &= \Phi_2 + k_1\gamma - k_s\gamma \\ d\gamma/dt &= \Phi_1 - k_1\gamma \end{aligned} \tag{2}$$

with

$$\Phi_1 = \frac{\varepsilon_1\alpha(1+\alpha)(1+\beta)^2}{L_1 + (1+\alpha)^2(1+\beta)^2}$$

$$\Phi_2 = \frac{\varepsilon_2\alpha(1+\alpha)(1+\gamma)^2}{L_2 + (1+\alpha)^2(1+\gamma)^2} \tag{3}$$

where α, β, and γ denote, respectively, the intracellular normalized concentration of ATP, 5'AMP and cAMP.

As in glycolysis, the analysis of the model shows that sustained oscillations of cAMP can occur around a nonequilibrium unstable stationary state (Goldbeter, 1975). Theoretical predictions compare with experimental observations as to the amplitude and waveform of the periodic cAMP signal. The analysis further shows that relay of a cAMP pulse can take place in the domain of parameter values corresponding to stable steady states.

Due to the phosphodiesterase reaction that transforms cAMP into 5'AMP, oscillations can still occur in the absence of ATP pyrophosphohydrolase (Gold-

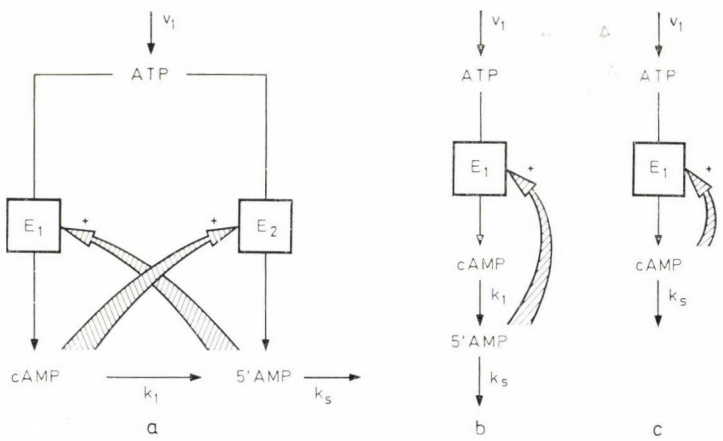

Fig. 4. Mechanisms for the periodic synthesis of cAMP in *D. discoideum* (see text and ref. Goldbeter, 1975). Enzymes E_1 and E_2 refer, respectively, to adenyl cyclase and ATP pyrophosphohydrolase. The rate constants k_1 and k_s, in (a) and (b), refer respectively to the phosphodiesterase and 5'nucleotidase reactions. In (c), constant k_s refers to phosphodiesterase. Dashed arrows indicate positive feedback. Scheme (c) is identical with the mechanism detailed in Fig. 1 for glycolytic oscillations; in the latter case, cAMP should be replaced by ADP, whereas enzyme E_1 would denote phosphofructokinase

beter, 1975). The positive feedback of 5'AMP on adenyl cyclase is thus sufficient for inducing periodic synthesis of cAMP, as indicated in Fig. 4b. It should be noted that a similar result obtains when adenyl cyclase is directly activated by cAMP (Fig. 4c). The latter regulatory effect could take place via the binding to the cAMP receptor on the cell membrane (Gerisch et al., 1975).

Scheme (c) in Fig. 4 is equivalent to the mechanism proposed for glycolytic oscillations (see Section 2). Indeed, scheme 4c is identical with the model represented with intermediate molecular steps in Fig. 1. This stresses the similarity of oscillatory mechanism in glycolysis and in cAMP synthesis. Both phenomena originate from the positive feedback exerted by a reaction product on an allosteric enzyme operating under nonequilibrium conditions.

The analysis of allosteric models for phosphofructokinase and adenyl cyclase shows that coherent behaviour in time and space may originate, far from equilibrium, from cellular metabolism. The physiological significance of metabolic dissipative structures is progressively coming to light. In *D. discoideum*, it has recently been shown that periodic cAMP pulses promote cell differentiation (Gerisch et al., 1975; Darmon et al., 1975). It is likely that oscillatory enzyme reactions may fulfil other functions, e.g. in synchronizing metabolic pathways or cell populations, in the origin of mitotic or circadian rhythms, etc.

It has been conjectured that spatiotemporal periodicities might play a role in the specification of positional information in embryogenesis (Goodwin, Cohen, 1969). In this respect, it is of interest to note that biochemical waves in the PFK model (Fig. 3) establish over supracellular dimensions comparable to those of morphogenetic fields. Chemical instabilities could also take part in the onset of polarity in morphogenetic fields (Babloyantz, Hiernaus, 1975).

Spatiotemporal organization or multiple steady-state transitions that occur under nonequilibrium conditions have also been discussed with respect to membran function (Blumenthal et al., 1975; Blumenthal, 1975), protein synthesis (Sanglier, Nicolis, 1976) and prebiotic evolution (Eigen, 1971; Prigogine et al., 1972).

Acknowledgements

I am grateful to Professors G. Nicolis and I. Prigogine for their interest in this work and for stimulating discussions. The author is Aspirent du F.N.R.S. (Belgium).

References

Babloyantz, A., Hiernaux, J. (1975) Bull. Math. Biol. *37* 637
Blumenthal, R. (1975) J. Theor. Biol. *49* 219
Blumenthal, R., Changeux, J. P., Lefever, R. (1970) J. Membr. Biol. *2* 351
Boiteux, A., Goldbeter, A., Hess, B. (1975) Proc. Nat. Acad. Sci. USA *72* 3829
Bonner, J. T. (1971) Annu. Rev. Microbiol. *25* 75

Darmon, M., Brachet, P., Pereira, da Silva, L. H. (1975) Proc. Nat. Acad. Sci. USA *72* 3163
Eigen, M. (1971) Naturwissenschaften *58* 465
Gerisch, G. (1968) Curr. Top. Dev. Biol. *3* 157
Gerisch, G., Hess, B. (1974) Proc. Nat. Acad. Sci. USA *71* 2118
Gerisch, G., Wick, V. (1975) Biochem. Biophys. Res. Commun. *65* 364
Gerisch, G., Hülser, D., Malchow, D., Wick, V. (1975) Phil. Trans. R. Soc. London Ser. B *272* 181
Glansdorff, P., Prigogine, I. (1971) Thermodynamic Theory of Structure, Stability and Fluctuations. Wiley, New York
Goldbeter, A. (1973) Proc. Nat. Acad. Sci. USA *70* 3255
Goldbeter, A. (1975) Nature *253* 540
Goldbeter, A., Caplan, S. R. (1976) Annu. Rev. Biophys. Bioeng. *5* 449
Goldbeter, A., Lefever, R. (1972) Biophys. J. *12* 1302
Goldbeter, A., Nicolis, G. (1976) Progr. Theor. Biol. *4* 65
Goodwin, B. C., Cohen, M. H. (1969) J. Theor. Biol. *25* 49
Hess, B., Boiteux, A. (1971) Annu. Rev. Biochem. *40* 237
Higgins, J. (1964) Proc. Nat. Acad. Sci. USA *51* 989
Monod, J., Wyman, J., Changeux, J. P. (1965) J. Mol. Biol. *12* 88
Nicolis, G. (1971) Adv. Chem. Phys. *19* 209
Prigogine, I. (1967) Introduction to Thermodynamics of Irreversible Processes. Wiley, New York, 3rd ed.
Prigogine, I., Nicolis, G., Babloyantz, A. (1972) Physics Today *25* 38
Roos, W., Nanjundiah, V., Malchow, D., Gerisch, G. (1975) FEBS Lett. *53* 139
Rossomando, E. F., Sussman, M. (1973) Proc. Nat. Acad. Sci. USA *70* 1254
Sanglier, M., Nicolis, G. (1976) Biophys. Chem. *4* 113
Sel'kov, E. (1968) Eur. J. Biochem. *4* 79
Shaffer, B. (1962) Adv. Morphogen. *2* 109
Shaffer, B. (1975) Nature *255* 549
Zaikin, A. N., Zhabotinsky, A. M. (1970) Nature *225* 535

Discussion

A. Kotyk: What is the explanation of the synchronous oscillation of a yeast population?

A. Goldbeter: The synchronization was found to be instantaneous. But we have no explanation.

A short discussion was about the name of this science: why it is called "thermodynamics" *(L. A. Blumenfeld)*, and why is it worth-while to study these oscillations *(R. Glaser)*. *A. Goldbeter* answered: the thermodynamic approach is very helpful from a conceptual point of view.

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Quantum Biophysics

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Next year, in 1977, will be celebrated the 50th anniversary of a major scientific event which took place in 1927: the publication of the first quantum-mechanical computation on a molecular system. This was the famous paper by Heiter and London on the hydrogen molecule, which provided the first acceptable description of the nature of the chemical bond. The work represented a major achievement and implied at that time heavy and arduous calculations.

50 years have past since that memorable event and, as in other branches of science and technique, the progress accomplished is enormous. Not only did the concepts of quantum mechanics penetrate deeply into all branches of *molecular sciences* but the actual computations have evolved to the point of being applicable and in fact applied to huge polyelectronic systems of the dimension of biological molecules. The turning point in these possibilities of practical applications was, of course, the advent of the modern electronic computers but the penetration of quantum-mechanical methodologies to structures and problems involving biological molecules and systems started somewhat earlier although naturally on a simpler level. The birthdate of quantum biophysics or biochemistry — at this fundamental level of investigation they are frequently undistinguishable — may be traced in fact to the late fifties.

One of the principal and rather distinctive characteristic of the quantum-mechanical approach to biophysics is its relative *polyvalence*. Thus all the other approaches and methodologies utilized in this field, e.g. X-ray crystallography, NMR spectroscopy, ultraviolet spectroscopy, thermodynamics, etc. provide information on some, frequently one precise property of the biosystems investigated. Quantum mechanics, on the other hand, by the virtue of its fundamental postulates, equations and methods deals with an extremely large number of facets of molecular reality. A quantum-mechanical computation, when properly managed, is able to produce simultaneous information on a large, very large, number of molecular properties, which experimentally need to be studied by different techniques and apparatus. Thus a suitably programmed computation may produce as direct output data simultaneous information on preferred conformations, associated dipole moments (value and direction), associated UV spectrum (predicted positions, intensities, polarizations of bands), associated ionization poten-

tials, electron affinities etc., etc. A particular advantage, which I consider quite major and unique of quantum-mechanics is to permit studies on inexisting structures and situations, which frequently enable a better understanding of the existing of such structures and situations. There is, however, also a disadvantage on this state of affair, namely that people then expect a quantum biophysicist to have an answer, frequently an instantaneous one, to all problems and questions which are preoccupying them. May I just say that if he does not, as it is most frequently the case, it is rather him than quantum mechanics which should be blamed.

I would like to indicate now, briefly in a highly summarized form, some of the principal lines of research in quantum biophysics.

The fundamental step which serves as a corner stone for more elaborate contributions and theories is the determination by appropriate computations of the electronic structure of essential biomolecules, by which we mean of their electronic distribution and of their electronic energy levels. This determination was done with more and more precision and accuracy in the course of time, as the methods of quantum-mechanical computations evolved from the simple Hückel theory, through the electron SCF method to the all valence electron methods (EHT, CNDO, PCILO) and *ab initio* SCF procedure, first without and afterward with configuration interaction. Directly related to these data is the determination and frequently prediction of a series of physicochemical properties of such molecules and systems, e.g. dipole moments, ionization potentials and electron affinities (electron donor and acceptor properties), excitation energies, polarizations of electronic transitions etc. These form then the basis for the investigation of the nature of the biophysical processes taking place in connection with these molecules or systems and/or for the elaboration of concepts and theories relevant to such processes. As examples of such achievement we may quote works carried out in such diversified fields and on such varied problems as the theory of intermolecular interactions, e.g. the mechanism of horizontal *vs* vertical (hydrogen-bonded *vs* stacking) interactions between the purine and pyrimidine bases of the nucleic acids and the nature of base complementarity, the vast problem of charge transfer and and charge transfer complexation between biomolecules, extending up to the function of the electron transfer enzymes and to the highly disputable and disputed question of the possible semiconductivity of biopolymers; the problem of the tautomeric structure and equilibria of biomolecules, rather difficult to investigate experimentally and relatively easy from the theoretical standpoint and which possibly has a direct relevance to the problem of mutations; problems of biospectroscopy: uv., visible, CD and of photo- and radiobiology where substantial theoretical contributions have been made to problems such as the mechanism of thymine photodimerization, the origine of the bathochromic shift of retinal in rhodopsine, the structure of excited states or photoinduced free radicals etc. etc.

A particular mention is perhaps deserved by the quantum-mechanical contribution to the NMR spectroscopy of biomolecules and biopolymers, in particular of nucleic acids and their constituents, through the theoretical evaluation of iso-

shielding curves or spin-spin coupling. This theoretical evaluation is, I believe, of an essential importance for the appropriate exploitation of experimental data (*vide* Prof. Ts'o).

A different type of contribution, which evolved only more recently because it needed the development of the all-valence or all-electrons methodologies, is represented by the quantum-mechanical exploration of molecular conformations. This has grown into an extremely important field of quantum biophysics, with moreover a very neat superiority over the so-called "empirical" procedures utilized by non-quantum biophysicists. Proteins, nucleic acids, phospholipids were and are extensively investigated from that point of view, as are also a large number os smaller molecules of both biological and pharmacological interests.

Finally the last and the latest line of quantum biophysical research is connected with the investigation of the effect of the environment on molecular structure and properties ("molecules in their environment"). This is an important step forward from the usual treatment of *free* or *isolated* molecules. It tends to replace the biological molecules at least partly in the medium in which their activity occurs. The most important aspect of this work is the inclusion of the solvent effect. Broadly speaking, efforts in this field are developing along two lines. Some proceed in the "traditional" way of dealing with the solvent problem through the use of a continuum model which tries in one way or another to account for the bulk effect of the surrounding medium. Others, and we are among those, try to develop a "discrete" treatment aiming to establish the individual effect of the medium molecule(s) upon the system studied. This is achieved through the utilization of the "supermolecule" model which combines, in a unique exploration, the molecular entities in interaction, such as the solute and the solvent molecules. Started with the detailed but fundamental exploration and elucidation of the nature of the hydrogen bond, investigated on relatively simple hydrogen bonded system, the procedure has evolved into a detailed examination of the hydration scheme of the majority of the fundamental biomolecules: hydration sites, location and number of hydration shells, structure of the bound water, effect of hydration upon molecular properties, say, conformation or tautomeric equilibria etc. In this important field, where experimental information is particularly scarce and imprecise, the computations of quantum biophysics are largely in advance on all other methodologies and on experiment. A somewhat similar situation prevails in connection with other types of environmental effects, also investigated by the supermolecule approach such as cation-binding to biological substrates. One must mention in this respect the important role played in the development of this line of research by the introduction of the notion of molecular electrostatic potential, which besides its direct significance for the study of proton affinity of biomolecules is liable to have important effects on the studies of biochemical reactivity.

These are only a few of the possible lines of thought and research through which quantum theories, methods and computations are trying to contribute to the development of biophysics. It is a difficult task in particular in as much as

it implies the convergence of such different disciplines as the abstract notions of quantum theory, the complexities of mathematics and computer programming, and the complexities of biological systems. It has, however, the advantage at going into the fundamentals of the problems studied and from that point of view it is bound to have a large development.

I try to illustrate some of the possibilities and aspects of quantum biophysics by resuming on a few figures some examples taken from the field of recent applications.

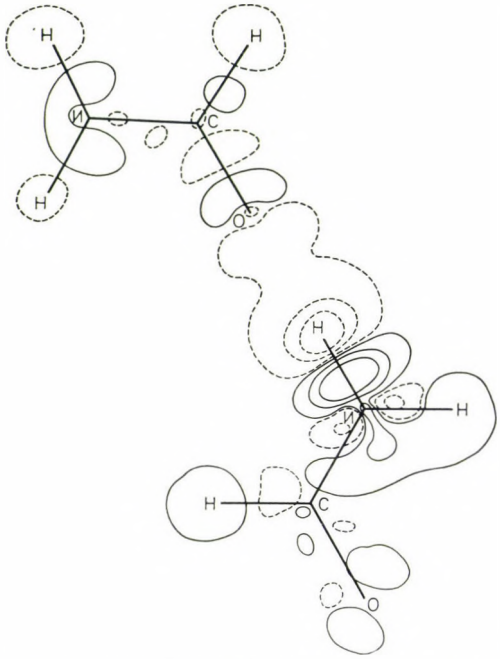

Fig. 1. Global density difference map for the formation of a hydrogen bond between two peptide bonds. Dashed lines represent regions of decreased density, full lines regions of increased density

Fig. 1 illustrates the present-day possibilities in computation of electronic structures. It represents a global electron density difference map for the hydrogen bond between two peptide linkages, computed by the "supermolecule" model within the *ab initio* SCF method (Dreyfus, Pullman A., 1970). Dashed lines represent regions inside which the density is decreased upon hydrogen bond formation, with respect to the sum of the densities of the isolated monomers, full lines represent the reverse situation. It is striking to observe the fine structure of the electronic distribution which shows that the electron density is in fact depleted in the region of the hydrogen bond, typically marked by the symbol – – – which suggests the contrary. This depletion has been confirmed recently by the combination of X-ray and neutron data (Griffin, Coppens, 1975).

Fig. 2 illustrates the results of *ab initio* SCF "supermolecule" investigations of the hydration scheme of the formamide molecule, the simplest representative of the peptide bond (Alagona et al., 1973; Pullman, Pullman, 1975). Thick lines indicate the most favorable positions for water fixation. Fig. 3 presents the same results for adenine. These theoretical results go far beyond the information that can be obtained presently experimentally, and represent the theoretical contributions to some aspects of the long debated problem of "bound water". Other aspects will be described later.

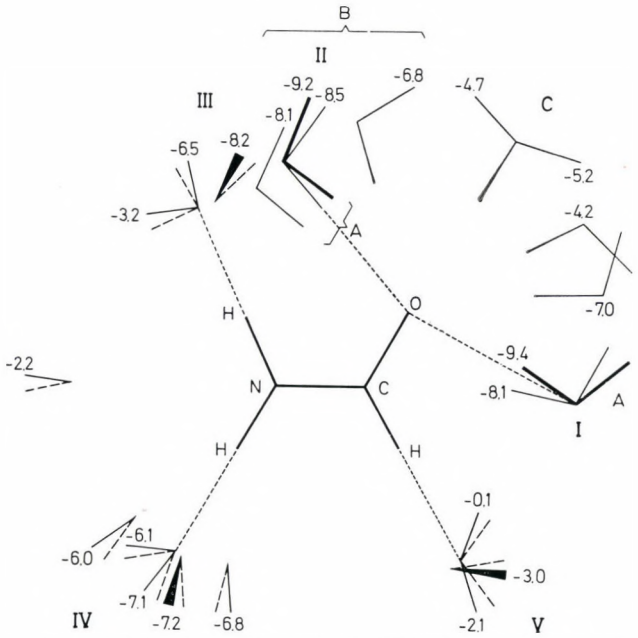

Fig. 2. Hydration scheme of the formamide molecule. Thick lines indicate preferred hydration sites. Energies in kcal/mole

Fig. 4, representing a molecule of phospholipid, symbolizes the possibilities of quantum-mechanical computations in the field of conformational studies. These molecules contain a very large number of degrees of freedom and up to very recently no X-ray crystal studies of them were available. The theoretical analysis by quantum-mechanical methods enabled the prediction of preferred values for all of them (Pullman, Saran, 1975). The superiority of these methods with respect to the so-called empirical ones may be indicated in conjunction with conformational studies concerning the phosphodiester linkage (Fig. 5), a particularly important grouping in the polar head of phospholipids and in nucleic acids. A typical "empirical" conformational energy map with respect to the torsion about the $P-O_{ester}$ bonds is presented in Fig. 6. It indicates a number of equivalent potential energy minima. Fig. 7 presents a quantum-mechanical

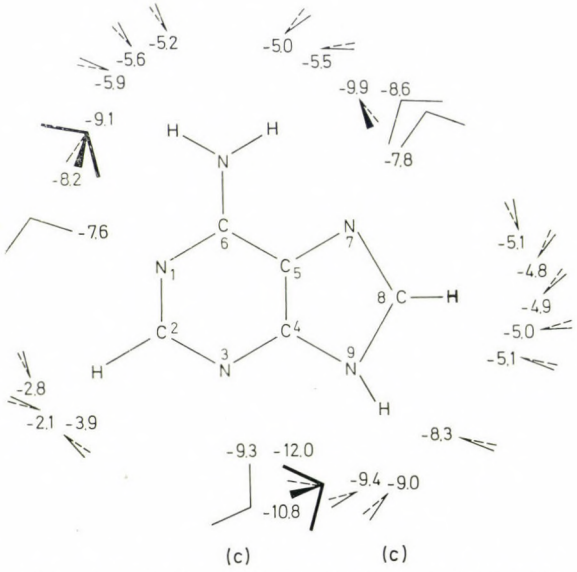

Fig. 3. Hydration scheme for adenine. Thick lines indicate preferred hydration sites. Energies in kcal/mole

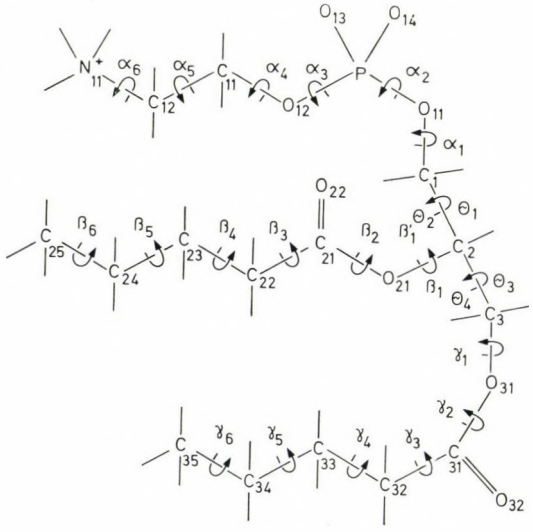

Fig. 4. Torsion angles in phospholipids

map (obtained by the PCILO method) which clearly distinguishes between the different minima and unambiguously predicts the *gg* conformation to be the preferred one (Pullman, B., Saran, A., in press).

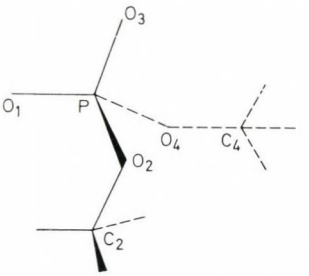

Fig. 5. Phosphodiester linkage, exemplified by dimethylphosphate (DMP^-)

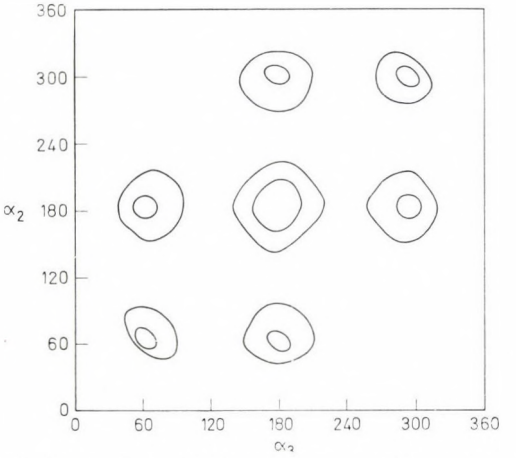

Fig. 6. Typical "empirical" conformational energy map for torsion about the $P-O_{ester}$ bonds

Let us come back to the problem of hydration and bound water. Fig. 8 presents a simplified representation of the hydration scheme of the phosphate group in its different OPO planes (Pullman et al., 1975). Many nearly equivalent hydration sites are available located essentially around the anionic oxygens (Fig. 9). The question may be raised about the possible influence of hydration on the conformational preference of the phosphodiester linkage. The answer to this question is given in Table 1, which shows how progressive filling up to the first hydration shell of the phosphate group, up to its completion (which occurs when 6 water molecules are fixed) tends to decrease the energy difference between the conformers.

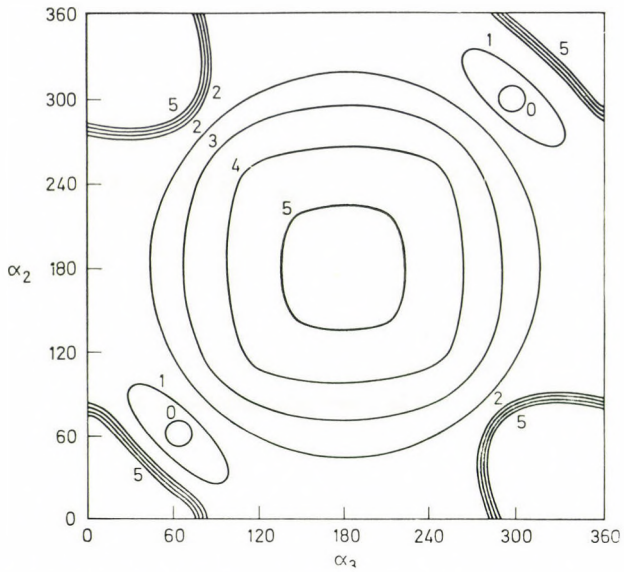

Fig. 7. Quantum-mechanical (PCILO) conformational energy map for torsion about the $P-O_{ester}$ bonds. Energies in kcal/mole with respect to the global energy minimum taken as energy zero

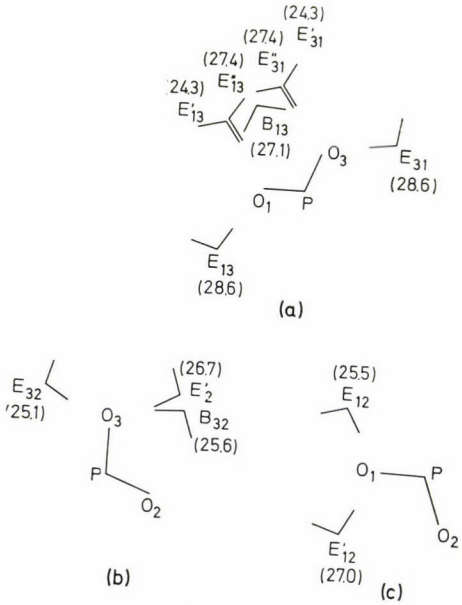

Fig. 8. Preferred hydration sites of DMP^- in the different OPO planes. Energies in kcal/mole

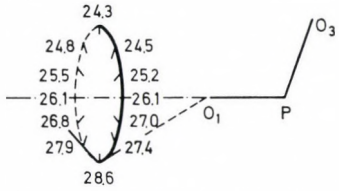

Fig. 9. Variation of hydration energies upon rotations of water about the $P-O_1$ axis

Table 1

Hydration of DMP⁻ (ab initio STO 3G)

(Energies in kcal/mole with respect to the gg form taken as energy zero)

H_2O	*gg*	*gt*	*tt*
DMP⁻	0	3.4	8.0
1		2.4 – 3.9	
2		2.9	
3		2.6	
4		1.2	
5		1.0_5	
6		0.0	1.7_5

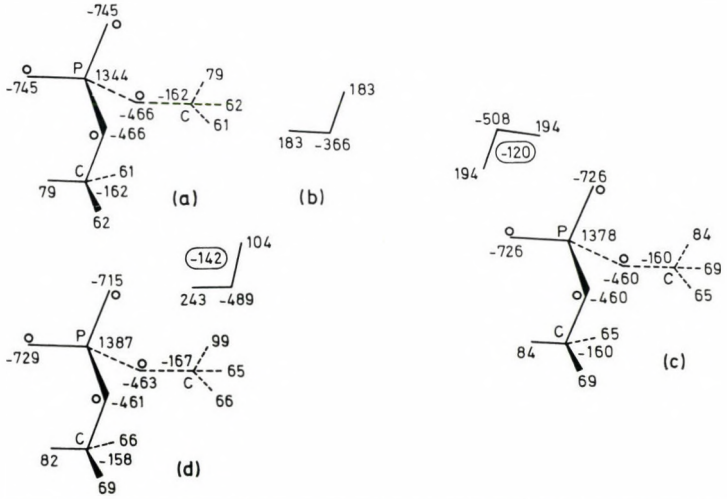

Fig. 10. Charge distribution (in millielectrons units) in (a) free DMP⁻, (b) free H_2O, (c) and (d) monohydrates of DMP⁻. Number in circles indicate the total amount of charge transferred from DMP⁻ to H_2O

Another fascinating problem concerns the structure of the bound water or more generally the perturbation brought in the electronic structure of the water and its substrate by their interaction, a long debated problem in biophysics. for which there is little experimental answer yet. An aspect of the theoretical investigations in this field is presented in Figs 10 and 11 which refer to the distribution of electronic charges. Figs 10a and 10b present the distribution of these charges in free dimethylphosphate (DMP^-) and water, respectively; Figs 10c and 10d represent the distribution in two different monoadducts. Fig. 11 gives the distribution of electronic charges in a heptahydrate of DMP^- in which 6 water molecules are in the first hydration shell and one in the second shell. Circled numbers indicate the amount of charge transfer (in millielectron units) from the phosphate to each water molecule. These studies point to the existence of two structured water layers around the phosphate (Pullman et al., 1975).

Similar studies have been carried out for cation binding. Fig. 12 indicates the preferred binding sites for mono- and divalent cations. Table 2 indicates the results of computations on the effect of cation binding upon the conformational preference of the phosphodiester linkage. It indicates that following the binding

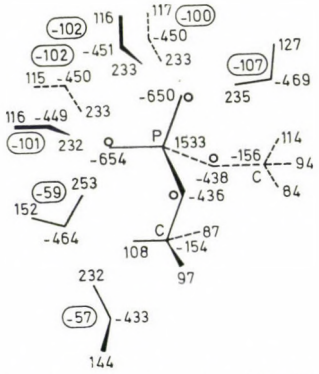

Fig. 11. Charge distribution in a hepathydrate of DMP^- (in millielectron units)

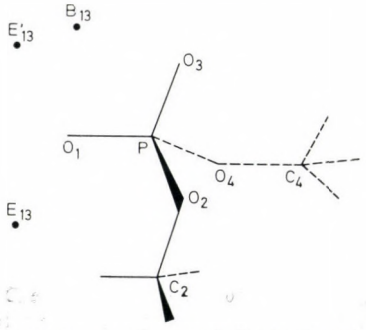

Fig. 12. Preferred (monovalent and divalent) cation binding sites

Table 2

Cation Binding to DMP⁻

Energies of conformers with respect of the gg taken as energy zero (kcal/mole)

		gg	*gt(tg)*		*tt*
Free DMP⁻		0	2.3		5.7
Cation At B_{13}	DMP⁻ . . . Na^+	0	4.4		10.0
	DMP⁻ . . . K^+	0	4.3		9.9
	DMP⁻ . . . Mg^{2+}	0	7.2		15.9
	DMP⁻ . . . Cal^{2+}	0	7.0		15.4
		gg	*tg*	*gt*	*tt*
Cation At E_{13}	DMP⁻ . . . Na^+	0	−4.7	5.1	1.6
	DMP⁻ . . . Ca^+	0	−10.9	8.5	−1.3

site and the nature of the cation, the fixation may or may not modify the intrinsic conformational preferences of DMP⁻.

Finally I would like to devote my last comment to the utilization in biophysics of the molecular electrostatic potential. This interesting recent concept (Scrocco, Tomasi, 1973; Pullman A., 1974) defines the global potential created in the space surrounding a molecule by the nuclear charges and the electron distribution. For a given wave function with corresponding electron distribution $\rho(i)$ and a set of nuclei α, the average value of the molecular potential at a given point P of space, $V(P)$ is:

$$V(P) = \sum_{\sigma} \frac{Z_\alpha}{r_{\alpha P}} - \int \frac{\rho(i)}{r_{Pi}} \mathrm{d}\tau_1$$

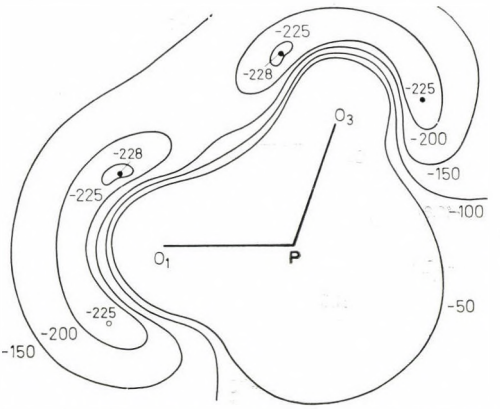

Fig. 13. Molecular electrostatic potential in the O_1PO_3 plane of DMP⁻

The molecular potential is numerically equal to the corresponding energy of interaction with a point positive charge and the usual representation employs isoenergy contours for such an interaction.

Fig. 13 presents such contours of the potential for the O_1PO_3 plane of the phosphate group, involving the two anionic oxygens (Berthod, Pullman, A., 1975). Strong energy minima are seen to exist around the oxygens. Fig. 14 shows the map obtained in the presence of a monovalent cation bound at position M^+ and Fig. 15 the map obtained in the presence of a divalent cation, placed at position M^{++} (Berthod, Pullman A., in press). The two maps illustrate the screening

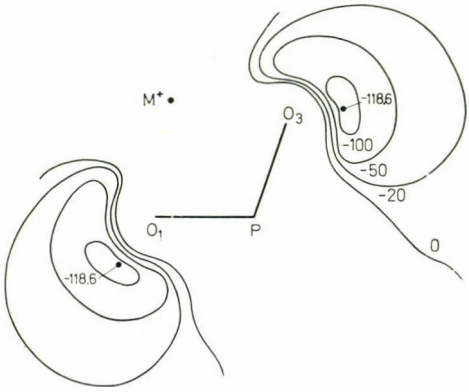

Fig. 14. Screening of the electrostatic molecular potential of DMP^- by a monovalent cation M^+

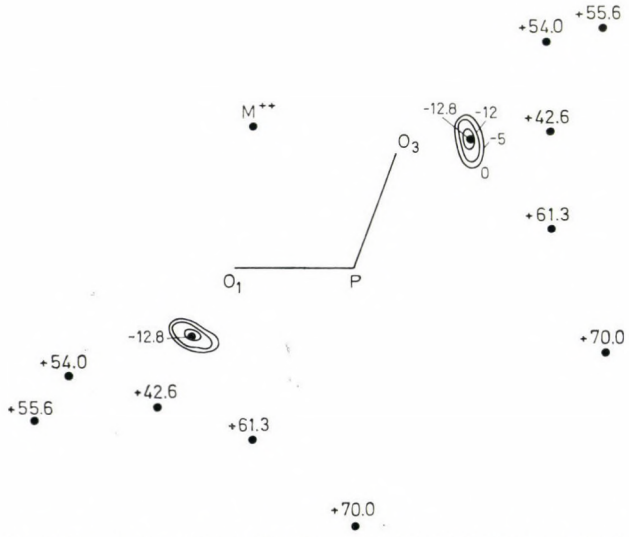

Fig. 15. Screening of the electrostatic molecular potential of DMP^- by a divalent cation M^{++}

of the electrostatic potential of the phosphate group by the cations and give a precise description of the details of this screening. They produce thus quantitative answers to a highly detailed biophysical problem, where again experimental results are very scarce.

It is, of course, impossible to present in such a short lapse of time the numerous aspect of quantum biophysics which are as broad and diversified as biophysics itself. What I essentially aimed at was to show you on a few selected examples some aspects of the enormous possibilities that are inherent in the quantum mechanical exploration of biophysical problems. Here is available a specific and powerful methodology which in many respects is complementary to or competitive with the best experimental techniques. The conjunction of both represents the best guarantee for a succesful unveiling and understanding of biophysics, extending beyond the molecular level into the realm of the submolecular.

References

Alagona, G., Pullman, A., Scrocco, E., Tomasi, J. (1973) Int. J. Peptide Protein Res., *5* 251
Berthod, H., Pullman, A. (1975) Chem. Phys. Lett. *32* 233
Berthod, H., Pullman, A. Chem. Phys. Lett., in press
Dreyfus, M., Pullman, A. (1970) Theoret. Chim. Acta *19* 20
Griffin, J. F., Coppens, Ph. (1975) J. Amer. Chem. Soc. *97* 3496
Pullman, A. (1974) in Chemical and Biochemical Reactivity, Proceeding of the 6th Jerusalem Symposium in Quantum Chemistry and Biochemistry (E. D. Bergman and B. Pullman eds) Academic Press, New York, p. 1
Pullman, A., Pullman, B. (1975) Quart. Rev. Biophys. *7* 505
Pullman, B., Pullman, A., Berthod, H., Gresh, N. (1975) Theoret. Chim. Acta, *40* 93
Pullman, B., Saran, A. (1975) Int. J. Quantum Chem. Quant. Biology, Symp. *2* 71
Pullman, B., Saran, A., Progress in Nucleic Acid Research and Molecular Biology, in press
Scrocco, A., Tomasi, J. (1973) Topics in Current Chemistry *42* 95

Discussion

I. C. P. Smith: The study of ion binding by NMR is complicated by the quadrupole splitting in the case of a particular nucleus, which depends on the electronic distribution around the nucleus. What the electrostatic potential around the nucleus could be? It would be useful to know from the point of view of bound water, too.

B. Pullman: This is an interesting question and a good base of cooperation.

P. O. P. Ts'o: Hydration problem of the phosphate has an outstanding significance. Another problem is the possible effects of the phosphate on the chemical shift.

B. Pullman: It is an interesting suggestion to deal with.

S. Maričić: There is a great difference between Na and Li in the hydration of DNA, 80 per cent, and 60 per cent, respectively. But these two different hydrations

bring about the same structural changes. Besides, the role of both the phosphate and the water has to be taken into consideration.

B. Pullman agrees with this remark; explicit calculation were carried out for different cations.

S. Maričić: What the conductivity of nucleic acids due to, protons or electrons?

B. Pullman: The semiconductivity of nucleic acids is more complicated than that of proteins. Electronic conduction may dominate, but the H-bonds might play a role.

K. L. Wierzchowski is interested in substrate enzyme complex structure computations.

B. Pullman: The electrostatic potentials of active sites of some enzymes was computed in the USA. (To V. F. Bystrov's question): The conformation of protein molecules has not been studied, perhaps in the future.

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The Biophysics of Nucleic Acids

(Summary)

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Less than three decades ago, nucleic acids, particularly DNA, have been identified as the genetic material which constitutes the physical basis for the information transferred from one generation to another. About a quarter of a century ago, the basic features of the double-stranded helix of DNA were elucidated, known as the Watson-Crick model. In this relatively short span of time, the research on nucleic acids has expanded rapidly, and always at the leading edge of science involving all areas of biology, much of chemistry, and even certain aspects of physics. At this juncture, the scientific development in nucleic acid research may indeed have a profound influence on the society, including the future development of our own race. A brief report on six topics in nucleic acid research, particularly those at the atomic and molecular levels, will be presented. Contributions from three major biophysical tools will be emphasized, partially because of their proven usefulness and partially due to the bias and limitations in the knowledge of this speaker. These techniques are X-ray diffraction, electron microscopy, and nuclear magnetic resonance. It can also be shown that the success of the application of these biophysical techniques depends heavily upon the supporting role of many areas of science, both in biology and in chemistry.

1. Spectacular advances have been made in the determination of the primary structure of nucleic acids, such as t-RNA, viral RNA and viral DNA. Sequences of up to a few thousand nucleotides in lenght have been determined and the methodology has been significantly improved and streamlined. Based on this sequence information, chemical and biochemical synthesis of known sequences have been accomplished; one notable example is the *E. coli* tyrosine suppressor structural gene. Ingenious techniques have been developed from many laboratories, particularly the group from Caltech, in visualizing the location of stretches of sequences by electron microscopy. This allows the orientation and geometrical location of the gene along viral and mammalian DNA.

2. X-ray diffraction techniques and nuclear magnetic resonance have made significant contributions to the elucidation of the structure and conformation of nucleic acids. These two techniques complement each other, with the X-ray diffraction techniques providing the atomic coordinates of the molecule in crystalline state, and with the nuclear magnetic resonance providing the information about the nucleic acids in solution at the atomic level and with information about

the dynamic aspects. Detailed descriptions have been obtained on bond lengths and bond angles of the monomeric units, dimers and polymers. Valuable information has been obtained from X-ray diffraction studies of the oriented fibers of nucleic acids and from the nuclear magnetic resonance studies of the short helices of nucleic acids in solution.

3. Specific emphasis should be placed on the research concerning the conformation and properties of the transfer ribonucleic acid (tRNA), the only nucleic acid currently known to have a well-defined biochemical function, suitable as a model system for the study of specific recognition of protein and nucleid acids. The atomic coordinates of the three-dimensional structure of yeast phenylalanine tRNA has been recently published by four groups. Nuclear magnetic resonance studies on this tRNA and others have also been made in providing precise information about the conformation of tRNA in solution. Recently, the three-dimensional structure of the aminoacyl tRNA synthetase has been obtained from X-ray diffraction studies. Considerable insights have been gained concerning the interaction between the tRNA and the synthetase, which is a vital step in the translation between the genetic code and the amino acid sequence of the protein.

4. A general review concerning the basic factors determining the properties and conformation of nucleic acids will be presented. The classical experiments describing the hydrophobic interaction stacking forces and the hydrogen bonding forces are reviewed together with the concept of how these forces are being brought together in unison in the interaction of nucleic acids. These cooperative interactions are best illustrated by experiments on the interaction of the monomers and the oligomers with their respective complementary polymers. Most recent experiments leading to a direct evaluation of the electrostatic forces in nucleic acid interaction will be described. These experiments are concerned with the properties of alkyl phosphotriesters of oligonucleotides, the charged groups of which have all been neutralized. The complex formations of these neutral oligonucleotide analogs with their complementary polynucleotides will be described. In addition, a report will be given on the biochemical effects of these analogs on the function of tRNA in amino acid acylation as well as their biological effects on protein synthesis in living cells. This research could lead to the development of compounds which may be employed to control the function of nucleic acids inside the living cell according to the sequence specificity of the nucleic acids.

5. A brief description will be given concerning the most recent advances in structure and function of chromatin.

6. A brief report will be given concerning the genetic engineering experiments which have introduced nucleic acids from one species of organism to another through chemical and biochemical means. Thus, it is now possible to transfer genetic materials, breaking through the barriers of species. The potential benefits and hazards, together with the recent efforts for developing guidelines for such research in the United States, will be reviewed.

In summary, the influence of nucleic acid research on the present society and the future development of the human race will once again be pondered.

Discussion

I. C. P. Smith proposed a Pan-European collaboration in the experimental work with NME because these are rather expensive ones.

B. Pullman put some stress on the carcinogenity of alkylating agents.

C. Helene asks if the presenter investigated the binding of this alkylated nucleic acids on RNA- or DNA-polimerase in order to find out if they are inhibitors on these enzymes. (The answer was "no").

A. Pullman speaks about the role of water on the stabilization of DNA. This stabilization may occur in different ways: by reducing repulsive forces between negative charges of phosphate groups or by increasing the stacking tendency of the bases. On the other hand it would be interesting that why some alkylating agents prefer nitrogen sites and others prefer oxigen sites.

A. Ehrenberg calls the attention that the spin labelling may change the structure of conformation of the labelled DRNA molecule.

B. Pullman: The interaction between drugs and nucleic acids are of two types: physical interaction and chemical – covalent – bounds. It does not seem likely that only physical interactions are responsible for the effectivity of drugs. If we take two quite similar drugs, many times one of them is carcinogenic and the other is not. There is no specific difference in their physical interactions, therefore a very specific binding of chemical type is needed to explain why is one of them carcinogenic and the other not.

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Environmental Biophysics

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When we whish to discuss environmental biophysics it is of particular importance to recall what we mean by biophysics. The important point is here, as I see it, that in biophysics we think of and work with biological problems as physicists. The problems have to be defined in physical terms and the results are discussed in a manner that is simultaneously of physical and biological relevance.

How do we then define environmental biophysics?

Historically biophysics started in many places as environmental biophysics, i.e. radiation biophysics. Radiation biophysics still is an important branch of biophysics, but only one out of many.

One could claim that in a wide sense all biophysics is environmental biophysics since all the biophysical and biochemical processes etc. of a living organism may be considered as its response to the combined physical and chemical influence of the environment. This view is, however, not very fruitful and for the present purpose it is useful to try to make certain distinction in order to obtain a classification of biophysics, particularly environmental biophysics, as a basis for our discussion.

Every species has developed a useful way to respond to the natural physical stimulation of the surrounding. This stimulation may be of a great variety: light, sound, gravity, pressure, high and low temperature, etc. The biophysics of such a physical stimulation and the induced response spans from the molecular to the physiological level. It starts with the phenomena of the receptor and transducer system and ends with the response or action resulting from the stimulation. The regulatory properties of one such system have been studied in detail by Reichardt (1976).

In simple organisms the whole process from stimulation to response is simpler than in more complicated ones where transmission of the information through nerves and the processing of the information in the brain are essential events. Even in the simplest systems the details of such a process are understood only in a very few cases.

This is an important area of continued research in the future. A better understanding of these processes on all levels will give us a basis for better understanding of the natural interplay between ourselves and the surrounding.

An interesting aspect of this is the possibility of developing new or improved technical devices to use when a sense, particularly vision or hearing, is malfunctioning or missing. But then we have left basic biophysics and entered medical technique.

As long as an external physical stimulation is below some limiting intensity threshold its effect on an organism is possible to be handled by its receptors. Also below a certain limit a specific external physical agent will not be harmful, but above the limit it will have deleterious effects which sometimes are irreversible. It is very important to find out where these limits are and the nature of the effects of the physical agent on the living organism and its molecules. These questions are synonymous with the more narrow and most often used definition of environmental biophysics. Let us look at some important research areas of this kind.

It is well known that ionizing radiation has genetic effects. It may give rise to mutations and initiate cancer formation. In order to elucidate these phenomena biophysical research has been carried out on several levels of very different complexity. Radiation chemistry of simple compounds is in several respects quite well developed. The direct effects on solid samples of the purine and pyrimidine bases have been studied very extensively. Investigation of the radiation damage in single crystals of such compounds has yielded very detailed information.

In our laboratory we have studied the radiation effects on the carrier of the genetic information, the macromolecular DNA itself. In this case no single crystals are yet available, but we have been fortunate to have access to large samples of well oriented DNA, obtained by a sophisticated wet-spinning technique developed by my colleague A. Rupprecht (1970). Experiments on oriented DNA containing some water of hydration have revealed that the primary radicals formed are most likely anionic radicals on thymine, $\dot{T}^-$, and cationic radicals on guanine, $\dot{G}^+$, (Gräslund, Ehrenberg, 1971; Gräslund et al., 1975a). These radicals are trapped when radiation is made at low temperature, and they may be studied at leisure at 77 K. Since most of the radicals are of these two types some kind of conduction of radical site along the DNA molecule must take place.

When temperature is increased the guanine cationic radicals disappear while the thymine anionic radicals take up protons under formation of the thymine hydrogen addition radical (Gräslund et al., 1976b): $\dot{T}^- + XH^+ \rightarrow \dot{T}H + X$. At higher temperatures the radical $\dot{T}H$ also disappears. These reactions have been studied in quite some detail and we have concluded that there is a fractional first (or pseudo-first) order reaction between spatially correlated pairs of $\dot{T}^-$ and a proton donor XH^+ with a distribution of activation energies (Gräslund et al., 1975b). Samples containing acridine dye molecules, known to be intercalated between the bases of DNA, have also been investigated (Gräslund et al., 1975c). It was found that the visible light absorbed by the dye molecules induced the same radicals, $\dot{T}^-$ and $\dot{G}^+$, in the DNA as the larger quanta of the ionizing radiation. For visible light it was shown that the radical induction was a two-photon process.

The mode of action of radiation sensitizers and inhibitors for different kinds of radiation effects in DNA is presently being investigated.

In other laboratories the direct effects of radiation on solid samples of amino acids and proteins have been studied quite extensively. The action of some protective agents, particularly sulphur containing compounds, has also been investigated.

Radiation effects on pure water have been studied in great detail. The induced changes in water may interfere with solute substances which are in this way affected indirectly. Some studies have been made on simpler molecules, but very little has as yet been done on the biological macromolecules.

Effects on living seeds of plants and bacterial spores have been studied in the past. The physical effect of the ionizing radiation resulted in free radical formation which was shown in case of the seeds to be correlated with the observed biological effects, e.g. growth inhibition and chromosomal aberrations (Sparrman et al., 1959). It was demonstrated that nitric oxide did scavenge the radiation induced radicals in the living seeds and simultaneously abolished the biological effects.

In the future it will be necessary to try to bridge the wide gap between these different levels of radiation biophysics. There are many important questions to deal with: What are the early primary effects on biological macromolecules in solution? What are the radiation effects on membranes and other supramolecular structures? What is the relative importance of direct and indirect effects in such systems? How will various factors such as temperature, viscosity, other solutes etc. influence these effects and alter the ratio between them? It is also very important to consider the natural DNA-repair processes. What are the physical and chemical factors that determine the effectivity and capacity of these repair processes? Can we find more effective ways of radiation therapy by means of discriminative agents for local sensitization or inhibition of the radiation effect?

Biophysicists also have to pay attention to the effects of less energetic radiation than the ionizing ones. Such radiation of high intensity is now coming into use technically in industries and also in households. I am thinking of laser light in the visible and infrared regions as well as the microwaves.

Today we are also exposed to a background irradiation of electromagnetic waves of a varying intensity at practically all frequencies. It is *a priori* feasible that some particular frequencies could have very specific effects on a living cell by for instance electric dipol resonance interaction with some critical molecular or supramolecular structure or with some transient reaction intermediate. To my knowledge no really serious experimental effort with modern technique has been made to try to find any such effect.

Similar specific effects on particular structures could perhaps also be expected from the absorption of ultrasound.

In the past few years it has been realized that low frequency sound, infrasound, from machines of various kinds could affect the well-being of men in a negative way. The biophysical and physiological basis of such effects should be explored.

Closely related to this is the area of biometeorology. Thunderstorms are

for instance powerful but transient sources of infrasound. Of specific importance are the physiological effects of electrical charges on ions and particles in the air. Negative charges are known to inhibit motion of cilia that clean and clear the respiratory channels. The dramatic increase in death rate during heavy smog attacks is believed to depend on this action of electrical charge. The physical and molecular details of this effects on the cilia are yet unknown. The phenomenon is probably closely connected with cooperative charge effects and ion binding to biological membranes and macromolecules.

One of the major problems in the modern society is the rapidly increasing exposure to chemicals of all kinds. This happens because of industrial production and environmental pollution from industries; because of chemicals in products that we use in the daily life and that we consume as food or medicine. There are also chemicals that we spread out in order to improve output from agriculture and forestry, etc.

The problem about the effects of these chemicals, their mode of action, how we can protect against their harmful effects, and which of them can be permitted and in what concentration, contains many elements that have a biophysical nature: Such a chemical first comes in contact with the membrane barrier of the cell or cells of a living organism. It either reacts directly with the membrane or it penetrates through it. How a foreign substance is incorporated into the supramolecular structure of the membrane or is transported through the membrane are biophysical problems of great importance.

Many of the foreign chemical compounds that enter the body of a human being are detoxicated by special hydroxylating enzyme systems, the cytochrome P-450 systems, situated in the membrane of the endoplasmatic reticulum of the cells. Several biophysical research groups are engaged in the investigation of the detailed molecular mechanism of these important reactions. This is a combined biophysical–biochemical problem. When a substrate molecule is taken up by the membrane the diffusional transport is changed from a three-dimensional to a two-dimensional process which means that the transport is speeded up. This may be visualized as an expansion of the active area of the cytochrome F-450 or an increase of the cross section of the reaction.

Of particular interest is that most of the so-called carcinogenic substances are harmless as such but become carcinogenic just because of the action of cytochrome P-450. The metabolites formed can in some way interact with DNA or some other part of the cell machinery so that cancer develops.

It is urgent to obtain the answers to questions such as: what property of a foreign substance determines how it interacts with membranes? How is a foreign substance transported through the membrane? How is it transported to other parts of the cell? How is it metabolized? How do the metabolites react with the different molecules of the cell? etc.

If we had all the answers to these questions we could, with the help of the theoretical chemists and the organic chemists, design chemicals that have specific physiological effects but are harmless in most other respects.

Practically all living systems on earth are directly or indirectly dependent on the property of green plants and photosynthetic bacteria to be able to utilize and convert the environmental radiation from the sun into energy-rich organic material. It appears as a matter of extreme importance to try to find out the electronic and molecular details of the various steps of this reaction. At certain points the compounds transporting electrons in the photosynthetic system are very similar to those participating in nitrogen fixation. An understanding of the physical and chemical principles of these reactions might lead to new ideas how we could convert solar energy into useful forms.

I have indicated that in a broad sense all biophysics is environmental in nature. When we think of environmental biophysics in the more restricted and more common way there emerge two types of questions:

One type concerns the action of physical factors in the environment and also protection against the harmful effects of these influences: How can biophysics help us to understand the biological action of physical and chemical factors in the environment? How can we learn to protect ourselves and other living systems against harmful influences? How can we control them?

The other type of questions deals with the possibility to use biophysical principles to make use of the environment: How can we apply biophysical principles and knowledge to make the best use of the environment? Can we do this for energy conversion on a useful scale? Can we do this in medicine?

I believe myself that the best way today to stimulate for results on both frontiers is to encourage basic research in biophysics in general. Parallel to this, study groups and symposia could be organized to focus the attention to possible applications. It is quite clear that several of these questions for their answer in every case requires efforts of expertness from more than one discipline, such as physiology, biochemistry and physics. Interdisciplinary collaboration will be needed. The experts are often working in different cities or different countries. Collaboration between them should be facilitated in every way.

References

Gräslund, A., Ehrenberg, A., Rupprecht, A., Ström, G. (1971) Biochim. Biophys. Acta *254* 172

Gräslund, A., Ehrenberg, A., Rupprecht, A., Ström, G., Crespi, H. (1975a) Int. J. Radiat. Biol. *28* 313

Gräslund, A., Ehrenberg, A., Rupprecht, A., Tjälldin, B., Ström, G. (1975b) Radiat. Res. *61* 488

Gräslund, A., Rupprecht, A., Ström, G. (1975c) Photochem. Photobiol. *21* 153

Reichardt, W. (1977) This issue

Rupprecht, A. (1966) Acta Chem. Scand. *20* 494

Rupprecht, A. (1970) Biotechnol. Bioeng. *12* 93

Sparrman, B., Ehrenberg, L., Ehrenberg, A. (1959) Acta Chem. Scand. *13* 199

Discussion

J. Jaz: The environmental biophysics is of vital importance. Considering this or the whole biophysics in general the relation of basic research and applied research is very important. The science policy-makers are not always scientists but economists first of all and the scientists have to influence them to find the correct relation of basic and applied research and to convince the governements that basic research needs a proper support in the framework of the national scientific research. This is an indispensable requirement for the development of the applied research.

J. Tigyi: Biophysics is a basic science by nature. Nowadays the politicians began to recognize the importance of the support of basic science, as it can be seen by studying the report of Brezhnev in the 25th Congress of the Soviet Communist Party. The Hungarian Acad. Sci. put to the front the development of basic research and the same is reflected by president Ford's new budget.

N. M. Emanuel: We are now at the beginning of our work in environmental biophysics to learn how to protect living organisms against pollution, radiation etc. We are sure our governments will give us all the supports to our work, our task is to find out what sort of concrete suggestions can be given from the side of the biophysics to contribute to the settling of these problems.

L. P. Kayushin raised the question that the microwaves can have an effect on living organisms.

R. Glaser speaks about the importance of energetic balance of ecosystems. Another point is the necessity of some dosimetric method to estimate the effect of electromagnetic waves in the radiofrequency range on workers who stay in the electromagnetic field during their work.

N. M. Emanuel: There are chemical protectors against radiation damage, e.g. if we treat a DNA preparation with an inhibitor of free-radical processes, the radiation decomposition of DNA can be suppressed. Such a radioprotector decreases the lethal effect of ionizing radiation. There are protectors against carcinogenic agents, as well.

J. Tigyi: The trace elements may play a special role in radiosensitivity, this is connected with semiconductivity of biological tissues.

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Protein–Nucleic Acid Interactions*

(Summary)

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Specific interactions between proteins and nucleic acids play a central role in the expression of genetic information and in replication. A very striking example is given by the *lac* repressor, a protein molecule ($M \simeq 150{,}000$) which controls the transcription of the three genes involved in the metabolism of lactose by *E. coli*. This protein binds strongly and selectively ($K_a \approx 10^3\ M^{-1}$) to a segment of DNA called *operator* which is only 25 base pairs long while the whole genome of *E. coli* is composed of 4 millions base pairs!

A general problem is raised by these very specific interactions: how can a protein molecule recognize a sequence of base pairs in DNA? is there a molecular code which allows this selective recognition? In order to be able to answer these questions we have to gain a better understanding of the following topics:

1. structural complementarity between the interacting molecules: what is the conformation of both the protein and the nucleic acid base sequence in the free and the complex state?

2. direct interactions between amino acid side chains or peptidic bonds on the protein side, and bases, phosphates or ribose (or deoxyribose) on the nucleic acid side. This includes electrostatic binding, hydrogen bonding, stacking and hydrophobic interactions.

3. indirect interactions between proteins and nucleic acid components mediated through a third species, a metal cation for example. Thermodynamic parameters for the binding of nucleic acids to proteins have been determined in a few cases (Table 1). They show that most of the free energy of association is due to a large positive entropy change. The origin of this large ΔS value has to be determined (hydration and conformational changes, role of metal cations . . .)

Kinetic parameters of complex formation between proteins and nucleic acids also have to be determined. They may reveal very interesting features. For example, the rate constant for the binding of *lac* repressor to *lac* operator is larger than that expected for a diffusion-controlled reaction. This observation probably reflects the fact that this reaction does not involve a three-dimensional

* A general discussion of these different problems can be found in a review paper published in Studia Biophysica (1976) *57* 211

diffusion but rather a one-dimensional diffusion. This is a very general problem since most reactions occurring in membranes will probably involve a two-dimensional diffusion in many cases.

Table 1

Thermodynamic parameters for some nucleic acid-protein complexes

System	K (M^{-1})	ΔH (kcal · $mole^{-1}$)	ΔS (cal · $mole^{-1}$ · deg^{-1})
Lac repressor + Lac operator Riggs et al., 1970)	10^{13}	+8.5	+90
tRNA Ileu E. *coli* + Ileu tRNA synthetase *(E. coli)* (Lam and Schimmel, 1975)	2.5×10^7	0	+34
tRNA val yeast + Ileu tRNA synthetase *(E. coli)* (Lam and Schimmel, 1975)	7×10^5	+8	+54
Photoreactivating enzyme 23 °C *(E. coli)* (Rupert et al., 1972)	6×10^8	+6.5	+60
Photoreactivating enzyme 23 °C (yeast) (Rupert et al., 1972)	1.8×10^{10}	+4.2	+60

Discussion

B. Pullman: From educational point of view we have to be careful what sort of terms we use. It is important to accentuate that all intermolecular forces are of the same kind fundamentally and one may not think that, e.g. H-bonds are something totally physically different, and do not forget that in this case the electrostatic component of the general intermolecular forces is dominant. So we must not use a loose descriptive language which can distort the ideas of young students about this topic. We speak this way for commodity reasons, but it is dangerous in the education.

L. A. Blumenfeld: The interaction between small and large molecules is a very important topic of molecular biology, but its classical physical approach may be wrong. The conformational changes must not be left out of consideration.

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The Investigation of Biopolymers in Dilute Solutions with X-ray Small-angle Scattering*

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In all scattering phenomena the scattering angles are inversely related to the dimensions of the scattering particles. As macromolecules are giant compared with the normally used CuK_α wavelength = 1.54 Å, we find the scattering of the particle as a whole at correspondingly small angles.

In this report we will confine ourselves to the important case of the diluted homodisperse solution of biological macromolecules. Here the intensities scattered from the single molecules simply add, so that the scattering curve (that is, the plot of the scattered intensity I versus the scattering angle 2Θ) gives a characterization of the single particle; we speak of p a r t i c u l a t e s c a t t e r i n g.

As the experimental method and the theory are well established and suitable computer programs for the evaluation are existing, it is no longer the privilege of a few highly specialized laboratories to perform small angle investigations.

Characteristic Parameters

The first step is to determine some characteristic parameters of the particles:

Radius of gyration. In the plot of ln I towards $(2\Theta)^2$ the innermost portion of the scattering curve is always a straight line, the slope of which, according to Guinier (1955), is directly related to the radius of gyration R of the particle:

$$\text{slope} = -KR^2; \qquad K = \left(\frac{2\pi}{\lambda}\right)^2 \cdot \frac{1}{3} \tag{1}$$

which is the square root of the mean square of the distance of all electrons from the center of gravity. I_t is a measure of the spatial extension of the macromolecule.

Radius of gyration of the cross section. The scattering curve of a particle, the one dimension of which is very large in comparison to its other dimensions, can be split into two factors, the cross-section factors I_c and the Lorentz factor

* For summarizing presentations of the method and applications especially in the field of biopolymers see references at the end of the text.

$1/2\Theta$. By multiplication of the scattering curve by 2Θ we get I_c. By the plot of $\ln I_c$ towards $(2\Theta)^2$ we get again from the slope of the curve in the innermost portion the radius of gyration of the cross section:

$$\text{slope} = -K' \cdot R^2; \qquad K' = \left(\frac{2\pi}{\lambda}\right)^2 \cdot \frac{1}{2} \tag{2}$$

In an elongated particle a simple relationship connects R, R_c and the length L:

$$L = \sqrt{12 \cdot (R^2 - R_c^2)} \tag{3}$$

Volume. The volume V of the solute particle can be calculated according to

$$V = \frac{\lambda^3}{4\pi} \frac{I_0}{\int_0^\infty I(2\Theta)^2 \, \mathrm{d}(2\Theta)} \tag{4}$$

(I_0 = scattered intensity at zero angle)

Area of the cross section. With elongated particles we get in analogous manner the area F of the cross section:

$$F = \frac{\lambda^2}{2\pi} \frac{[I(2\Theta)]_0}{\int_0^\infty I(2\Theta)^2 \cdot \mathrm{d}(2\Theta)} \tag{5}$$

Molecular weight M. For its determination we need: I_0/P_0 (P_0 = intensity of the primary beam) and the partial specific volume $\bar{v}_1$ of the solute.

Molecular weight per unit length. With elongated particles, using instead of I_0 the zero intensity of the cross-section factor $I(2\Theta)_0$ we find the mass per unit length $M/1$ Å.

In many cases the above parameters alone give valuable informations as to the morphology of biological macromolecules. As an impressive example we choose the enzyme glutamate dehydrogenase. It was known, that with increasing concentration an association of the monomeric particle occurs. From the outer portion of the scattering curves we find independent from the concentration, always the same cross-section factor (Fig. 1), which gives $R_c \approx 30$ Å. Therefrom we conclude that the association occurs in the length direction. On the other hand we find in the inner portion of the scattering curve with increasing concentration an increasing radius of gyration, from which we calculate with (3) the length L of the particles. The linear increase of the independently determined molecular weight with the length (Fig. 2) confirmes the picture, that particles with $R_c \approx 30$ Å associate in the length direction.

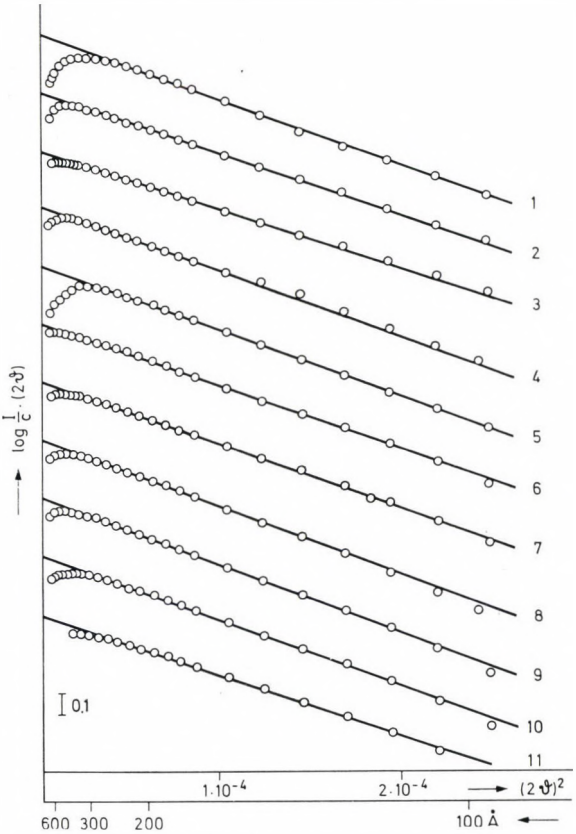

Fig. 1. Guinier plot of the cross section factors of glutamate dehydrogenase solutions. The concentrations c of the solutions vary from 1 to 33 mg/ml (Sund, H., Pilz, I., Herbst, M. (1969) Eur. J. Biochem. *7* 517)

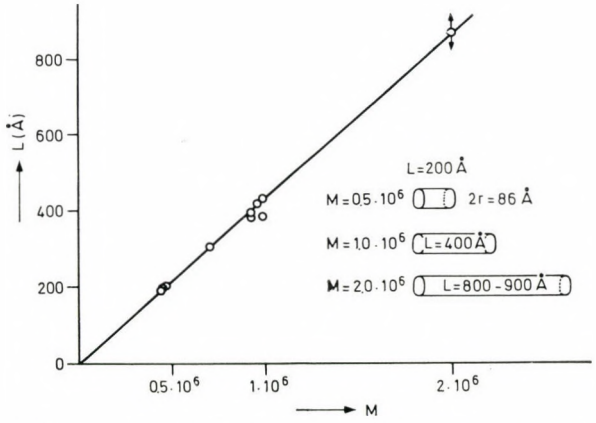

Fig. 2. Length L of glutamate dehydrogenase particles in phosphate buffer, pH 7.6, as a function of molecular weight M (Sund, H., Pilz, I., Herbst, M. (1969) Eur. J. Biochem. *7* 517)

Overall Shape

The next step is the determination of the overall shape of the particle by the attempt to find out with the help of a collection of scattering curves of three-axial bodies (like ellipsoids, elliptic cylinders, prisms, hollow spheres and so on) that one, which coincides best with the experimental scattering curve. When there is a coincidence with respect to the main maximum then the selected shape can be considered as a reasonable approximation to that of the real particle.

As an example Fig. 3 compares the scattering curve of chymotrypsinogen-A with the theoretical curves of ellipsoids of revolution.

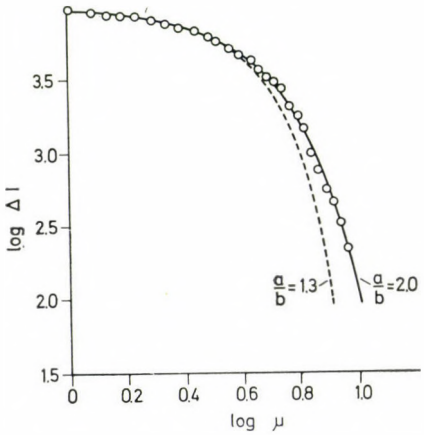

Fig. 3. Comparison of the experimental scattering curve for a 1.832 per cent solution of chymotrypsinogen-A (○) with the theoretical scattering curve for ellipsoids with an axial ratio of 2 : 1 (full line) and 1.3 : 1 (dashed line) (Krigbaum, W. R., Godwin, R. W. (1968) Biochemistry *7* 3126)

A second example is the above discussed glutamate dehydrogenase. Fig. 4 shows – in the plot of $\log I$ towards $\log hR$; $h = (2\pi/\lambda) \sin 2\Theta$ – the experimental curves of those three types of particles which are sketched in Fig. 2, in comparison to the theoretical curves of circular cylinders. We recognize the good coincidence of this shape determination with the dimensions calculated according to (3). This shows, that the model, sketched in Fig. 2, is correct.

A certain refinement often can be derived from the first side maximum, in that its intensity reflects the degree of hollowness. Fig. 5, for example, shows the first side maximum in the hight relative to I_0 for different hollow cylinders. The experimental scattering curve of hemocyanine fits best into this row of curves and we realize that the quotient of inner and outer radius r_i/r_a equals 0.45.

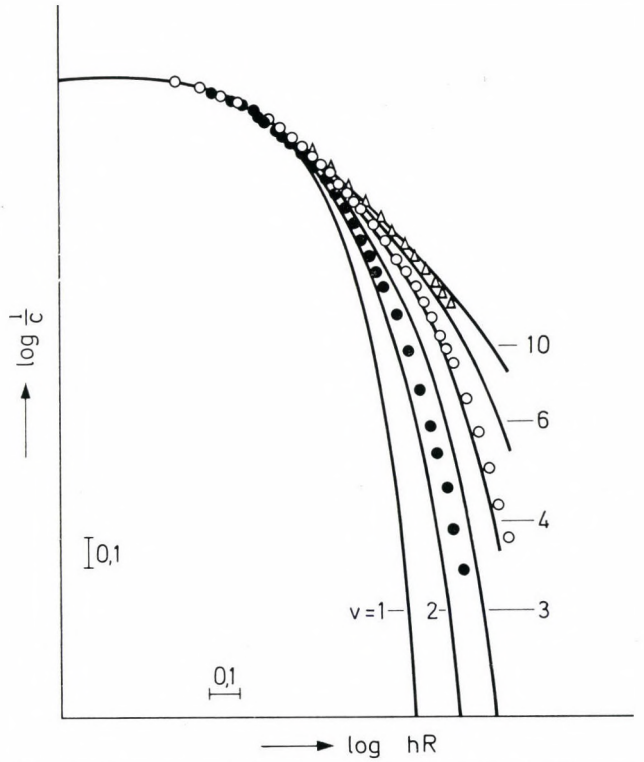

Fig. 4. Theoretical scattering curves of circular cylinders (full lines). The ratio v of length (L) to the diameter ($2r$) of the cylinder ($v = L/2r$) is 1, 2, 3, 4, 6 and 10. Experimental scattering curves of glutamate dehydrogenase particles of the following approximate molecular weight: 0.5×10^6 (×), 1×10^6 (○), 2×10^6 (△). $h = 2\pi \times (2 \sin \Theta)$ (Sund, H., Pilz, I., Herbst, M (1969) Eur. J. Biochem. *7* 517)

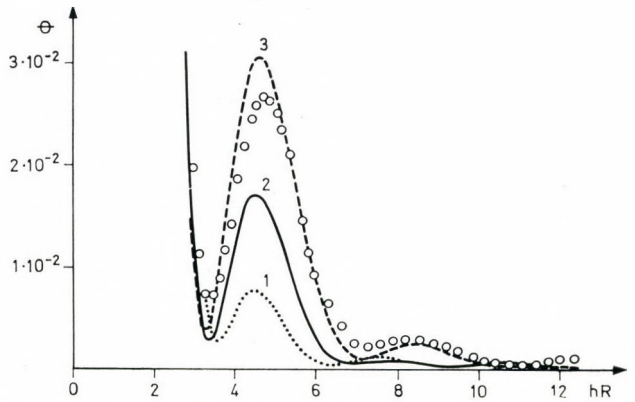

Fig. 5. Comparison of the experimental scattering curve of hemocyanin in H_2O (○) with theoretical scattering curves of circular hollow cylinders with the following quotient of the inner radius r_i to the outer radius r_a: Curve 1: full cylinder; curve 2: $r_i/r_a = 0.3$; curve 3: $r_i/r_a = 0.5$ (Pilz, I., Kratky, O., Moring-Claisson, I. (1970) Z. f. Naturfsch. *25b* 600)

Refinement of the structure determination

However, while the parameters R, V and M (resp. R_c, F and $M/1$ Å with rodlike particles) represent a final result of the investigation, the overall shape is only the first step in the elucidation of the structure and a refinement has to yield additional informations. For the way of the refinement we select the following cases as representative examples:

1. In the case of *γGI-immunoglobulin* the main maximum of the cross section factor is composed of two branches (Fig. 6) and it is not possible to interprete this finding with the help of simple elongated particles, so not even an approximative overall shape could be determined by comparison with the collection of theoretical scattering curves. On the basis of biochemical evidence the scattering curves of composite bodies formed by several ellipsoids have been calculated. As shown in Fig. 6 the arrangement number 9 gives a theoretical scattering curve, which fits best the experimental one.

It was further interesting to investigate the specific interaction of the γGI-immunoglobulin with haptens; while the molecule as a whole showed a contraction of its longest dimension by several per cent, the dimensions of the isolated polypeptide chains are unaltered upon binding the haptens at their outer ends. From this finding it can be concluded that the conformational change found with the antibody as a whole occurs in the hinge zone between the single chains.

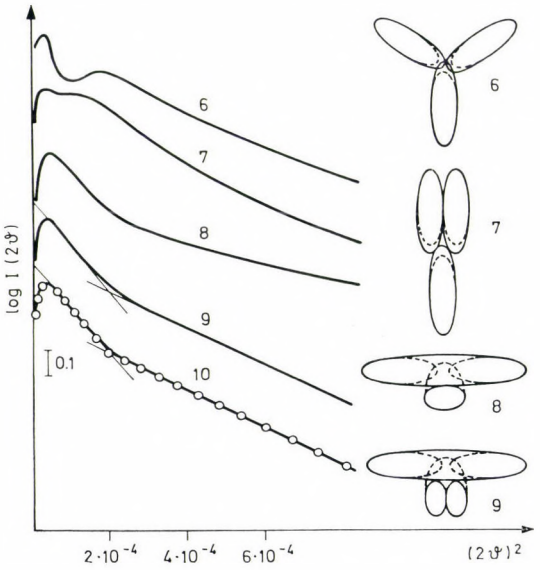

Fig. 6. Comparison of the calculated cross section curves for various models (6—9) with the experimental cross section curve of γGI-immunoglobulin (10). The dashed lines correspond to the ellipsoids equivalent to the individual fragments of the immunoglobulin. (Pilz, I., Puchwein, G., Kratky, O., Herbst, M., Haager, O., Gall, W. E., Edelman, G. M. (1970) Biochemistry *9* 211)

2. With *t-RNA*^{Phe} the main maximum of the scattering curve showed similarity with theoretical curves of rotational cylinders (Fig. 7). A much better fit, however, can be achieved with the theoretical curve of the composite body shown in Fig. 7, which therefore obviously represents an optimal approximation to the real shape. Indeed some years later on the way of crystal structure analysis a very similar shape could be found.

3. With particles which show approximately spherical symmetry it is possible by a simple Fourier-inversion to derive the radial electron density distribution. Together with the known chemical composition it is often possible to establish a structural model of the particle. The *lipoproteins* give good examples of this sort, one of these is shown in Fig. 8. The success of such work depends strongly on the exact measurement of several side maxima (Fig. 9) which is an experimentally difficult task.

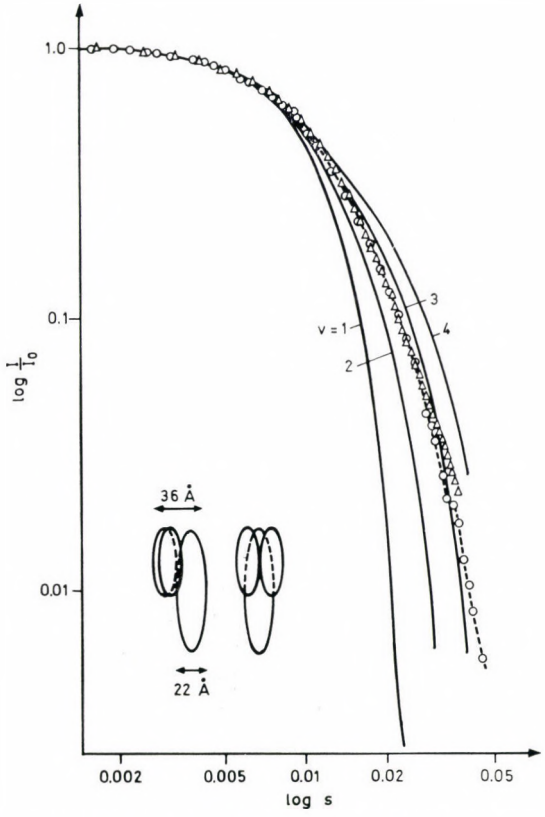

Fig. 7. Experimental scattering curve (○) of tRNA^{Phe} (yeast) at 17° compared with the theoretical scattering curves of rotational cylinders of various axial ratios v (closed lines) and with the theoretical curve of the model formed from three ellipsoids (△) in log—log plot. $s = 2 \sin \Theta/\lambda$ (Pilz, I., Kratky, O., Cramer, F., von der Haar, F., Schlimme, E. (1970) Eur. J. Biochem. *15* 401)

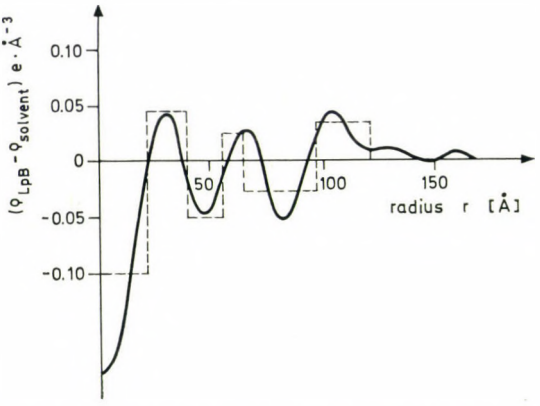

Fig. 8. Radial electron density distribution of human low density lipoprotein (Laggner, P., Müller, K., Kratky, O., Kostner, G., Holasek, A. (1976) J. Colloid Interface Sci. *55* 102)

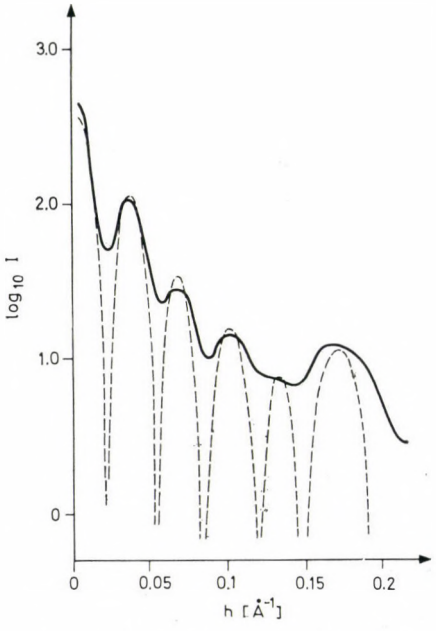

Fig. 9. Experimental scattering curve (———) of human low density lipoprotein and theoretical scattering curve (- - - - - -) for the continuous radial electron density distribution (Fig. 8) obtained by Fourier-transformation (Laggner, P., Müller, K., Kratky, O., Kostner, G. Holasek, A. (1976) J. Colloid Interface Sci. *55* 102)

4. When a particle has a substructure of the sort, that it is composed of many sphere-like subunits of the same size, the distances of neighbouring units can be found from certain minima to be expected for theoretical reasons at relative large angle. This information can be used to build a model. *Hemocyanin* is an interesting example of this type (Figs 10 and 11).

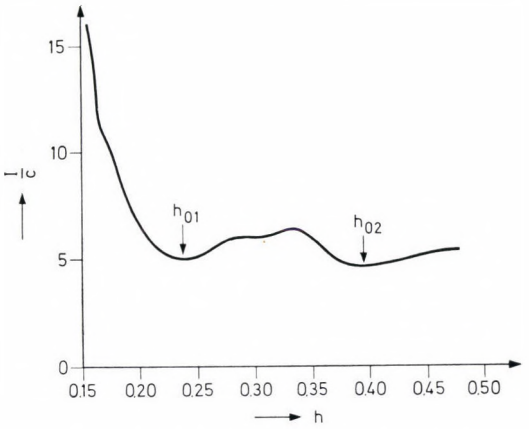

Fig. 10. Outer portion of the experimental scattering curve of hemocyani ($h = \frac{4\pi}{\lambda} \sin \Theta$)
(Pilz, I., Glatter, O., Kratky, O. (1972) Z. f. Naturfschg. *27b* 518)

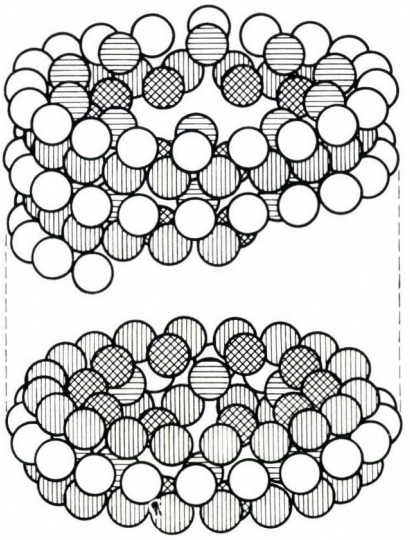

Fig. 11. Model of hemocyanin built up of 360 identical subunits (Pilz, I., Glatter, O., Kratky, O. (1972) Z. f. Naturfschg. *27b* 518)

5. In certain cases an extremely precise measurement of solely one parameter can help to clear up the type of a reaction. We mention the saturations of the apoenzyme of yeast glyceraldehyde-3-phosphate dehydrogenase with the coenzyme NAD to the holoenzyme. The volume turned out to be a valuable parameter to follow this interaction. First it was found that the complete saturation caused a contraction of the volume of 7 per cent. Now the question arose, as to the mechanism of the saturation: is it a sequential or an allosteric one. In the first case a linear relationship between volume and degree of saturation is to be expected, in the second case any deviation from the linearity can occur. Fig. 12 shows the results which are clearly in favour of an allosteric mechanism, in best coincidence with the results of kinetic measurements.

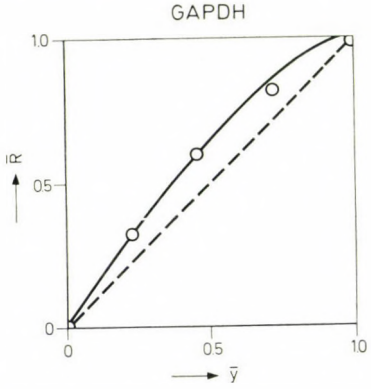

Fig. 12. Volume contraction of glyceraldehyde-3-phosphate dehydrogenase as a function of saturation with NAD. $\bar{y}$ = degree of saturation with NAD; $\bar{R}$ = degree of relative volume contraction. The diagonal represents the predictions for the simple sequential mechanism of cooperative binding. ○, Experimentally observed degree of contraction, expressed as a fraction of maximal contraction. (Durchschlag, H., Puchwein, G., Kratky, O., Schuster, I., Kirschner, K. (1971) Eur. J. Biochem. *19* 9)

References

Summarizing presentations

Beeman, W., Kaesberg, P., Anderegg, J. W., Webb, M. B. (1957) Handbuch der Physik *32* 321 ed. by S. Flügge, Springer Verlag

Brumberger, H. (1967) "Small-Angle X-Ray Scattering" Proceedings of the Conference held at Syracuse University, June 1965, Gordon and Breach, Science Publ. New York, London, Paris

Guinier, A., Fournet, G. (1955) Small Angle Scattering of X-Rays, J. Wiley and Sons Inc., New York and Chapmann and Hall, London

Kratky, O. (1963) Progress in Biophysics *13* 105 Pergamon Press, Oxford, London, New York, Paris

Kratky, O., Pilz, I. (1972) Quarterly Reviews of Biophysics *5* 481

Pressen, H., Kumosinski, T. F., Timasheff, S. N. (1973) in: Methods of Enzymology, Vol. XXVII, Enzyme Structure Part D, ed. by Hirs C.H.W. and Timasheff, S. N., Acad. Press, New York, London p. 151

Pilz, I. (1973) in: Physical Principles and Techniques of Protein Chemistry, Part C, Acad. Press, Ind., New York and London, p. 141

Discussion

L. A. Blumenfeld: What is the time resolution of the small angle scattering method?

O. Kratky: Between one week and 0.5 second. It depends on the concentration, intensity of X-rays and the electron density differences.

C. Helene suggests that neutron diffraction studies are useful to find out the structure of biomolecules.

I. P. C. Smith (to L. P. Kayushin). What was the temperature of mitochondria you worked with?

L. P. Kayushin: It was 20 °C. The temperature was not relevant, but the presence of oxygen was absolutely necessary.

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Biospectroscopy of Free Radicals

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In 1929 L. Michaelis discovered free radicals during oxidation of biologically important compounds what allowed him to make a conclusion that unpaired electrons are necessary stages of biological oxidation reactions (Michaelis, 1929). This discovery of L. Michaelis was a remarkable landmark in the development of biophysics. It is notable that conception of free radical character of oxidation of chemical substances in gas phase allowed N. Semenov and S. Hinshelwood in their time to create theory of chain reaction (Semenov, 1934, 1958). Unfortunately the level of state of biology in the thirties-fourties did not allow the ideas of L. Michaelis to be fully developed. Even after the investigations of B. Commoner (Commoner et al., 1954) and W. Gordy et al. (Gordy et al., 1955) in the fifties who applied electron paramagnetic resonance (EPR) for the studies of biological processes, many of the biologists kept on thinking sceptically to the idea that free radicals played an important role in biological reactions. The creation of free radicals theory of oxidation of hydrocarbons in liquid state by N. Emanuel (Emanuel, 1959) which served as ideological base for further development of conception about free radical character of many biological processes dates from the same time. 15–20 years passed until thanks to the efforts of a number of scientists it was shown that free radicals take an important role in different biological phenomena (Harold, et al., 1972; Kayushin, 1970; Blumenfeld, 1974). Still it is necessary to stress that even at present it is not yet clear when free radicals and paramagnetic centers in biological processes are conditioned by chain character of reactions.

Both in chemistry and biology the development of investigations of free radicals and paramagnetic particles and of biophysics of electron transport in general were to great degree dependent on a successful application of EPR spectroscopy. The phenomenon discovered by E. Zawoisky in Kasan in 1944 was at once used by chemists and is widely applicated in biophysics nowadays.

Free radical conception combined with EPR method should be the bases for studying the problem of transformation and accumulation of energy in biological systems. To a great degree the elementary bioenergetic processes are conditioned by phenomena of the origin, transport and localization of protons and electrons in biological structures. The latters suffer conformational changes that

in combination with electron and proton transfer demonstrate the basic elements of the work of the bioenergetical machine. In this case the EPR spectroscopy is greatly strengthened by using of other radiospectroscopic methods (NMR, ENDOR, CPN, laser EPR), high speed spectroscopy and quantum biochemical calculations. The application of these methods in biology necessitates a wide application of computers not only for solving calculations but for making the collection of spectral information automatic as well that leads to the origin of an applied branch of biophysics – to biospectroscopy of free radicals.

The problem of free radicals in biology is connected with the problem of bioenergetics first of all. One can say that existence and dynamics of free radicals represent material bases of bioenergetical conversion. It is not enough known how living organisms transform the absorbed light energy into energy of chemical bonds. We do not know in what way energetical transformation accompanying the biooxidation of different substances result in ATP formation. What is the intimate nature of biological catalysis of ATP hydrolysis during muscle contraction and nerve conductivity? What is occurring in cells while they absorb strong photons of γ-irradiation or are involved in cancerogenesis? In a number of cases free radicals initiating bioenergetical reactions being unusual for normal state of biological objects play an important role in the origin and development of diseases of organism. Lately in a number of laboratories the investigations in this field of biophysics were directed to the study of the nature and role of free radicals in oxidative metabolism of living systems to find reactions where free radicals take part and to develop the methods of their activation and depression.

Several examples can be represented here to demonstrate the advantage of free radical approach for studying bioenergetical reactions. The effectiveness of application of EPR method for studying electron transport of biological systems is conditioned by the fact that a number of basic steps of electron transfer in subcellular structures (mitochondria, chloroplasts) are carried out with participation of free radicals and paramagnetic centers that is by molecular fragments containing unpaired electrons (Harold et al. 1972). Apparently the mitochondria accomplishing phosphorylating oxidation represent the ideal object for such ESR studies and in fact this method has allowed to discover and identify a number of unknown paramagnetic centers of respiratory chain of mitochondria. The whole group of iron-sulphur proteins was discovered and characterized by using ESR-spectroscopy (Harold et al. 1972). It is known, however, that mitochondria contain some other respiratory carriers which take an active part in electron transport and can be investigated by EPR spectroscopy method. The measurement of respiration and phosphorylation as well as the recording of EPR absorption were made in the water medium at room temperature. EPR signals of free radicals of mitochondria were measured in special flow system. The flow speed was selected in such a way that the medium was saturated by oxygen. During the transition of mitochondria in the state of active oxidation coupled with phosphorylation the increase of EPR absorption of free radicals was observed. It was not connected with the change of rate of electron transport along a chain of respiratory carriers

and was conditioned by production of energized intermediates at the stages before the ATP is formed.

It was shown by different methods that two phosphorylation sites are localized in the cytochrome system (Chance, 1972). Various ideas are available accounting for the mechanism of oxidative phosphorylation which take into account changes in different characteristics of cytochromes. One of the main function of the cytochrome system is to convert the flow of hydrogenes into a flow of single electrons. Essential reconstruction of electron density at aromatic amino acid residues of cytochrome *c* during oxidation-reduction was shown by NMR method (Sibeldina, 1975). It can be said in particular that in a given case the molecule of cytochrome *c* acts as an electron-molecular trigger regulating the electron transport. It appears natural to assume that the coupling of substrate oxidation with production of ATP is accomplished by inpaired electrons whose transport is provided by the cytochrome system. According to the proposals the unpaired electrons must be obligatory stages involved in ATP synthesis (Brzhevskaya et al., 1966, 1967).

The ATP hydrolysis (as well as synthesis) should include free radical state. Enzymatic ATP hydrolysis was studied by some independent methods allowing to discover free radicals (Kayushin, 1973).

We have given here several examples of effective use of radiospectroscopic approaches for solving the problems of bioenergetics.

Apparently it should be said here that in the frames of science which nowadays is called molecular biology the most exciting problems as mechanism of transformation of energy in biological processes have not been touched (Szent-Györgyi, 1971). Very often we stress the importance of structural aspect in the development of biophysical conceptions. This is true and this is a point where biophysics comes into contact with molecular biology, cytology and with other branches of biology.

About 40 years ago nobody could imagine the whole significance of ATP. Already in those years A. Szent-Györgyi, A. Lenindger, V. Engelhardt tackled to this problem. They were the first who realized the role of this universal energetical substance. It is obvious that the final understanding how energetical machine of cell works can be obtained by the junction of two directions – structural and energetical. This is why serious attention should be paid to the investigation of bioenergetical problems in physical aspect. It is highly true as A. Szent-Györgyi and B. Pullman noticed that it is necessary to penetrate into electron structure of biologically important molecules realizing that their structure and properties are conditioned by the behaviour and interaction of electrons. The principal difference of biophysics from other branches of biology is the variety of physical methods it possesses. Everything that biophysics possesses: optical spectroscopy, NMR and EPR, everything that allows to control the electronic structure of molecules should help us to solve problems of bioenergetics.

References

Л. А. Блюменфельд: Проблемы биологической физики, Наука, Москва, 1974.

Бржвеская О. Н , Каюшин Л. П., Кондрашова М. Н., Неделина О. С., Шекшеев Э. М : Биофизика 11, 1076, 1966.

Бржвеская О. Н., Каюшин Л. П., Маринов Б. С., Неделина О. С., Шекшеев Э. М.: Биофизика 12, 39, 1967.

Chance, B. (1972) FEBS Letters, 21

Commoner, B., Townsend, J., Pake, G. (1954) Nature *174* 689

Эмануель Н М.: Проблема управления ценными реакциями окисления углеводородов в жидкой фазе. В кн. »Окисление углеводородов в жидкой фазе«. Сб. статей, М., АН СССР, 1959.

Gordy, W., Ard, W. B., Shields H. (1955) Proc. Nat. Acad. Sci. USA *41* 983

Swartz, H. M., Bolton, J. R., Borg, D. C. (1972) Biological Applications of Electron Spin Resonance, John Wiley and Sons, Inc.

Kayushin, L. P. (1973) The Role of Free Radicals in Regulation of Bioenergetic Processes. IVth Internat. Biophysics Congress Symposial Papers, Pushchino

Каюшин Л. П., Львов К. М., Пулатова М. К.: Исследование парамагнитных центров облученных белков. Наука, Москва, 1970.

Michaelis, L. (1929) J. Biol. Chem. *84* 777

Семенов Н. Н.: Цепные реакции М—Л, ОНТИ, Госхимтезиздат, 1934.

Семенов Н. Н.: О некоторых проблемах химической кинетики и реакционной способности, Издательство АН СССР, Москва, 1958.

Сент-Дьерди А.: Биоэлектроника. Изд. МИР, Москва, 1971.

Sibeldina, L. A., Kayushin, L. P., Kutushenko, V. P. Okon, M. S., Lasarevy, A. V., Chekulaeva, L. N. (1975) Conformational Change of Cytochrome *c* upon the Oxidation-reduction in Solution. Soviet-French symposium on the physical chemistry of proteins and peptides.

Atlas der Biochemie

Übersichten zum Intermediärstoffwechsel

Von Dr. med. RAINER LASEK

Medizinisch-poliklinisches Institut der Karl-Marx-Universität, Leipzig
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Preparation and Characterization of N^G-Mono-, Di- and Trimethylated Arginines

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A simple procedure is described for the synthesis of N-guanidino methylated ariginines. The yields of all methylarginines exceed those obtained by earlier procedures. Trimethylarginine — an amino acid not found in natural sources — has also been synthesized with this technique. Studies with arginine specific dicarbonyl reagents showed that methylation of guanidino-groups decreases the reactivity of methylarginines, only monomethylarginine reacting at a rate comparable to that of arginine.

Introduction

N-Guanidino-methylated arginines have recently been found in a variety of proteins (Paik, Kim, 1975), particularly in myelin basic protein (Baldwin, Carnegie, 1971) and myosin (Reporter, Corbin, 1971). It has been demonstrated that methylation of arginine residues occurs subsequent to synthesis of these proteins (Morse et al., 1975). The free methylated arginines found in serum and urine are apparently released by hydrolysis of methylated proteins (Kakimoto, Akazawa, 1970).

Although many authors speculated on the biological significance of protein methylation (Nickerson, 1973; Segal, 1973) the function of this post-translational modification remains unknown. Assessment of the possible role of arginine methylation requires a detailed knowledge of the chemical and physical properties of methylarginines. To facilitate investigations in this area we have worked out a simple method for the preparation of MMA, sDMA, and uDMA. The same synthetic procedure was also employed to prepare a new amino acid – N^G-trimethylarginine. Chemical and analytical properties of the methylarginines have been studied and a rapid amino acid analyzer system has been elaborated for the resolution of all methylarginines.

Abbreviations used: MMA, N^G-monomethylarginine; sDMA, symmetrical or N^G, N'^G-dimethylarginine; uDMA, unsymmetrical or N^G,N^G-dimethylarginine; TMA, N^G, N^G,N'^G-trimethylarginine.

Experimental section

Materials

N,S-Dimethyl-, N, N, S-trimethyl-, N,N',S-trimethyl- and N,N,N',S-tetramethylisothiouronium iodide were prepared according to a described method (Schenck, 1911). L-Ornithine and L-ornithine hydrochloride (Reanal, Budapest), flavianic acid (Pfalz & Bauer), 1,2-cyclohexanedione (Fluka), 2,3-butanedione, glyoxal (Sigma), phenanthrenequinone and phenylglyoxal (Aldrich) were commercial products.

Methods

A. *Synthesis of N^G-methylarginines.* Reactions were carried out in solutions 1 molar for ornithine and the isothiouronium salt. The haloacids present in the reaction mixture were neutralized with equivalent amount of sodium hydroxide. The reaction was followed by amino acid analysis and the reaction was found to be complete after 5–10 days at room temperature or after 5–10 hours at 100 °C. At the end of the incubation period the pH of the reaction mixture was brought to 4 with hydrochloric acid and the solution was applied to a Dowex 50×2 column (200–400 mesh) in the H^+ form. The column was washed with water until the eluate was neutral then washed with 0.2 N ammonium hydroxide. The amino acids were eluted with 1.5 N ammonium hydroxide and the elution of the amino acids was monitored by amino acid analysis. The fractions containing pure methylated arginine were pooled and concentrated under reduced pressure.

1) N^G-Monomethylarginine (MMA) acetate: L-Ornithine (10 mmoles) and N,S-dimethylisothiouronium iodide (10 mmoles) were dissolved in 10 ml 1 N sodium hydroxide. After incubation for 5 hours in a water bath at 100 °C the solution was cooled and pH brought to 4 with HCl. MMA was isolated from the reaction mixture by ion-exhange chromatography as described above. The residue obtained by drying the MMA containing fractions under reduced pressure was dissolved in acetic acid and the MMA acetate was crystallized by adding ethanol and acetone. The vacuum-dried crystals weighed 2.0 g (80% yield). M. p. 194–196 °C.

$C_7H_{16}O_2N_4 \cdot C_2H_4O_2$ (mol. wt. 248 · 28)

Calculated: C, 43.54; H, 8.12; O, 25.78; N, 22.57

Found: C, 43.60; H, 8.20; O, 25.71; N, 22.59

2) N^G,N'^G-Dimethylarginine (sDMA) hydrochloride: L-Ornithine hydrochloride (10 mmoles) and N,N',S-trimethylisothiouronnium iodide (10 mmoles) were dissolved in 10 ml 2 N sodium hydroxide and the solution was left at room temperature for nine days. The sDMA was purified by ion-exchange chromatog-

raphy as described above, and the syrup obtained by drying in vacuo was dissolved in concentrated HCl and crystallized by the addition of alcohol and acetone. The vacuum-dried sDMA.2HCl weighed 2.1 g (76% yield). M. p. 233–234°C.

$C_8H_{18}O_2N_4 \cdot 2HCl \cdot 1/4\ C_2H_6O$ (mol. wt. 286.70)

Calculated: C, 35.61; H, 7.56; O, 12.56; N, 19.54; Cl, 24.73

Found: C, 35.32; H, 7.60; O, 12.33; N, 19.70; Cl, 24.91

3) N^G,N^G-Dimethylarginine (uDMA) hydrochloride: L-Ornithine hydrochloride (5 mmoles) and N,N,S-trimethylisothiouronium iodide (5 mmoles) were dissolved in 5 ml 2 N sodium hydroxide and the solution was left at room temperature for 10 days. The uDMA was purified as described above then dissolved in concentrated HCl and crystallized by the addition of ethanol and acetone. The dried crystals of uDMA hydrochloride weighed 0.9 g (75% yield). M.p. 200–202 °C.

$C_8H_{18}O_2N_4 \cdot HCl \cdot 1/3H_2O$ (mol. wt. 244 · 73)

Calculated: C, 39.26; H, 8.10; O, 15.25; N, 22.90; Cl, 14.49

Found: C, 39.21; H, 8.11; O, 15.50; N, 22.75; Cl, 14.33

4) N^G,N^G,N'^G-Trimethylarginine (TMA) flavianate: L-Ornithine hydrochloride (3 mmoles) and N,N,N',S-tetramethylisothiouronium iodide (3 mmoles) were dissolved in 6 ml of 1 N sodium hydroxide and incubated at 100 °C for 10 hours. The reaction mixture was subjected to column chromatography as described above. The free base of TMA was dissolved in water (2 ml) and 6 mmoles of flavianic acid was added in 2 ml water. The diflavianate of trimethylarginine was washed with water and dried to yield 1.05 (41% yield). M. p. 214–216 °C.

$C_9H_{20}O_2N_4 \cdot 2\ C_{10}H_6O_8N_2S$ (mol. wt. 844.74)

Calculated: C, 41.23; H, 3.82; O, 34.09; N, 13,27; S, 7.59

Found: C, 41,12; H, 4.00; O, 34.05; N, 13.16; S, 7.68

B. *Isolation of methylarginines from human urine.* Human urine (100 ml) was applied to a column (2.5 × 30 cm) of Dowex 50W × 2, NH_4^+ form, 200–400 mesh. The column was washed first with one bed volume of water then with one bed volume of 0.1 M ammonium bicarbonate pH 8.0, thus removing the acidic, neutral and imidazole-containing amino acids as monitored by high-voltage paper electrophoresis of the eluate at pH 1.9 and pH 6.5. The aliphatic basic amino acids were eluted with 5% ammonia solution and the elution sequence was followed by high-voltage paper electrophoresis. Fractions containing basic amino acids were dried and the residues were hydrolyzed with 6 N HCl in sealed ampules at 110 °C for 24 hours before amino acid analysis. This step was necessary to ensure

that small basic peptides present in urine do not interfere with the identification of minor amino acids (methylarginines are not destroyed by this treatment).

C. *Analysis of methylated arginines.* Amino acid analyses were performed on a JEOL JLC-5AH automatic amino acid analyzer. Methylarginines were resolved using a 0.8×10.5 cm column of Durrum DC6A resin equilibrated with 0.35 N sodium citrate pH 5.00. Amino acids were eluted with the same buffer at a flow rate of 1.22 ml/min; the column temperature was 52.5°C.

Fixion 50-X8 (Na^+) chromatoplates coated with Dowex 50-X8 type resin (Chinoin-Nagytétény, Budapest, Hungary) were also used for the separation of methylated arginines. The developing buffer, pH 6.0, contained citric acid hydrate (100 g), sodium hydroxide (60 g) and 14 ml of concentrated hydrochloric acid in 1 liter solution. Chromatography was performed at room temperature, chromatograms were stained with ninhydrine.

D. *Reaction of methylarginines with guanidino-specific reagents.* Modification of methylated arginines with 1,2-cyclohexanedione was performed as described for arginine (Patthy, Smith, 1975). Analysis of the products of the reaction was achieved by paper electrophoresis at pH 1.9. The extent of reaction was estimated by cutting out the ninhydrine-stained spots of products and unreacted arginine, eluting the stain with 50% ethanol and reading the absorption at 500 nm. Treatment of methylarginines with other dicarbonyls and estimation of reaction was performed in a similar manner.

Phenanthrenequinone-test was carried out according to a described method (Yamada, Itano, 1966). Electropherograms containing 50–50 nmoles of arginine derivative were dipped into the alkaline-ethanolic solution of phenanthrenequinone and the dried paper-sheets were scanned under ultraviolet light.

Results and discussion

We have worked out a simple method for the uniform preparation of N-guanidino methylated arginines (Bajusz, Patthy, 1974). The advantage of the present method derives from the recognition that it is not necessary to protect the α-amino group of ornithine in the reaction with the methylisothiouronium salt. This possibility was suggested by the early observation that reaction of free lysine or ornithine with active esters led to the formation of the ω-acyl derivative (Schallenberg, Calvin, 1955). Since preparation of the copper complex of ornithine – a step common to previous methods of methylarginine synthesis (Kakimoto, Akazawa, 1970; Corbin, Reporter, 1974) – could be omitted, the purification of products is simplified, a single step of ion-exchange chromatography being sufficient for the isolation of pure methylarginines. The yields of MMA (80%), sDMA (76%) and uDMA (75%) are higher than those obtained with earlier methods (Corbin, Reporter, 1974, MMA: 52%; sDMA: 37%; uDMA: 26%).

The same preparation scheme was also used to extend the list of methylated arginine derivatives – to synthesize N^G-trimethylarginine.

A rapid amino acid analyzer program was developed for the resolution of all arginine derivatives (Fig. 1). Methylarginines could also be separated from each other on Fixion 50-X8 chromatoplates at pH 6.0. The R_f values for MMA, sDMA, uDMA and TMA were 0.20, 0.16, 0.15 and 0.12, respectively. The chromatographic behaviour of synthetic methylarginines was identical in both systems with that of the corresponding natural methylarginines isolated from urine.

Analysis of human urine showed roughly equal concentrations of the two dimethylarginines (sDMA: 37 μM, uDMA: 41 μM) and lower concentration of

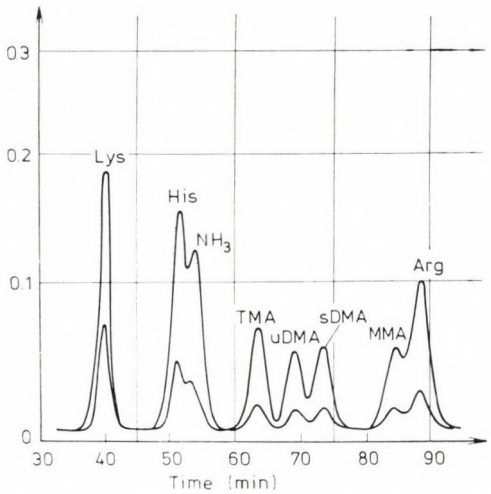

Fig. 1. Separation of methylated arginines by automatic amino acid analysis. A standard amino acid mixture containing 10 nmoles of each amino acid plus 5–5 nmoles of methylated arginines was chromatographed on a JEOL JLC-5AH amino acid analyzer as described in the Experimental section.

Table 1

Reaction of methylated arginines with dicarbonyl reagents

Reactions were carried out in 0.15 M borate buffer, pH 9.0, with 2 mM arginine derivative and 40 mM of dicarbonyl compound at 25 °C, and per cent reaction was determined after the time indicated.

	Cyclohexanedione (60 min)	Butanedione (15 min)	Phenylglyoxal (60 min)	Glyoxal (240 min)
Arginine	80	90	85	70
MMA	40	45	50	40
uDMA	5	5	30	10
sDMA	0	0	10	0
TMA	0	0	5	0

arginine (12 μM). No evidence was obtained for the presence of monomethylarginine or trimethylarginine even after isolation of basic amino acids from large volumes (100 ml) of urine, although this enrichment procedure should have permitted the detection of these amino acids if present in urine at a concentration of 1 μM.

N^G-Methylation strongly affects the reactivity of the guanidino-group towards various dicarbonyls (Table 1). Only MMA reacted at a rate comparable to that of arginine, while the di- and trimethyl derivative resist modification. It is of some analytical importance that the phenanthrenequinone reagent used for the visualization of arginine containing peptides on chromatograms, fails to give fluorescent products with any of the methylated arginines.

References

Bajusz, S., Patthy, A. (1974) Hung. Pat. No. 165543
Baldwin, G. S., Carnegie, P. R. (1971) Science *171* 579
Corbin, J. L., Reporter, M. (1974) Anal. Biochem. *57* 310
Kakimoto, Y., Akazawa, Sh. (1970) J. Biol. Chem. *245* 5751
Morse, R. K., Vergnes, J. P., Malloy, J., McManus, I. R. (1975) Biochemistry *14* 4316
Nickerson, K. W. (1973) J. Theor. Biol. *40* 507
Paik, W. K., Kim, S. (1975) Adv. Enzymol. *42* 227
Patthy, L., Smith, E. L. (1975) J. Biol. Chem. *250* 557
Reporter, M., Corbin, J. L. (1971) Biochem. Biophys. Res. Commun. *43* 644
Schallenberg, E. E., Calvin, M. (1955) J. Am. Chem. Soc. *77* 2779
Schenck, M. (1911) Archiv. der Pharmazie *249* 478
Segal, H. L. (1973) Science *180* 25
Yamada, Sh., Itano, H. A. (1966) Biochim. Biophys. Acta *130* 538

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 12 (3), pp. 197—205 (1977)

Activation of D-Glyceraldehyde-3-Phosphate Dehydrogenase by L-α-Amino Acids and Related Compounds

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The time course of the reaction catalyzed by D-glyceraldehyde-3-phosphate dehydrogenase shows a simple monophasic character when the reaction is started with the enzyme. However, started with D-glyceraldehyde-3-P in Hepes, Tris or triethanolamine buffers at pH 8.5, the reaction has complex kinetics. Preincubation of the enzyme with D-glyceraldehyde-3-P abolishes the initial lag of NADH formation. All L-α-amino acids, SH compounds and chelating agents tested change the complex kinetic curves into monophasic ones.

A group of L-α-amino acids including serine, glycine, alanine, arginine, phenylalanine, glutamic acid and lysine activates the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Aspartic acid has the strongest activating effect. Histidine, some SH compounds (2-mercaptoethanol, dithiothreitol, cysteine) and chelating agents (EDTA, o-phenantroline, 8-hydroxyquinoline) produce maximum curves in plots of enzymic activity *vs* effector concentration. The activating effect of all effectors used is readily observable only if the reaction is started with D-glyceraldehyde-3-P, whereas the activating effect is negligible if the reaction is started with the enzyme.

Similar kinetic curves are obtained with substrate at saturation and with D-glyceraldehyde-3-P partly replaced by glycine, EDTA or o-phenantroline.

It is suggested that compounds containing at least one oxygen, nitrogen or sulphur atom attached to each of two adjacent carbon atoms may be bound by the regulatory center of the enzyme and induce similar conformational changes in glyceraldehyde-3-phosphate dehydrogenase.

Introduction

Different effects of metabolites on the activity of glyceraldehyde-3-phosphate dehydrogenase (EC. 1. 2. 1. 12) from mammalian muscle have been established. An excess of NAD^+ and P_i inhibits (Nagradova, 1958; Keleti, Telegdi, 1959; Batke, Keleti, 1968; Ovádi et al., 1972), while that of D-glyceraldehyde-3-P activates the enzyme (Batke, Keleti, 1968). The substrate analogue, D-glyceraldehyde, is also an activator if added in excess (Keleti, 1966). The product, NADH (Batke, Keleti, 1968), and ATP in excess (Stancel, Deal, 1969; 1969a, Constantinides, Deal, 1969; Oguchi et al., 1973) or in stoichiometric amount (Ovádi et al., 1971, 1972) are inhibitors of the enzyme.

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Rabbit muscle (Dimitrieva et al., 1974) and Chlorella (Tomova et al., 1972, 1973) glyceraldehyde-3-phosphate dehydrogenases are activated by L-α-amino acids. However, β-alanine, γ-amino butyric acid and proline have no effect. Some SH compounds and chelating agents are also activators. The extent and the rate of enzyme activation depend on the preincubation of the enzyme with the substrates glyceraldehyde-3-P or glyceric acid-1,3-diP.

The present paper deals with the effect of L-α-amino acids and related compounds on the kinetics of glyceraldehyde-3-phosphate dehydrogenase from mammalian muscle. Part of these data were presented at the 9th FEBS Meeting, Budapest (Tomova et al., 1974).

Materials and methods

The experiments were carried out with four times recrystallized pig muscle glyceraldehyde-3-phosphate dehydrogenase (Elődi, Szörényi, 1956). D-glyceraldehyde-3-P was prepared from fructose-1,6-bisphosphate according to Szewczuk et al. (1961), D,L-glyceraldehyde-3-P obtained from Boehringer, Mannheim, and NAD from Reanal (Budapest). Effectors and buffers were of reagent grade. All solutions were made with distilled water, further deionized with Elgacan C 115.

Enzymatic activity with glyceraldehyde-3-P, NAD^+ and P_i as substrates was determined by Warburg's optical test at pH 8.5 and 20 °C according to Keleti and Batke (1965) but in 0.025 M Hepes buffer. The specific activity of the enzyme was expressed in international units (IU = μmoles NAD^+ reduced/min/mg protein) by using $E^{340\ nm}_{NADH} = 6.22 \times 10^3 M^{-1} cm^{-1}$ (Horecker, Kornberg, 1948).

Firmly bound NAD^+ was removed by charcoal treatment (Velick et al., 1953), which increased the absorbance ratio A_{280nm}/A_{260nm} from 1.05 to 1.90–1.95. Protein concentration was determined on the basis of light absorbance at 280 nm by using the extinction coefficients $A^{1\%}_{1\ cm} = 10$ and 9 for the holo- and apoenzyme, respectively (Cseke, Boross, 1970).

Spectrophotometric measurements were carried out in an Option PMQ II or Durrum-Gibson stopped-flow spectrophotometer.

Results and discussion

The reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase in Hepes, Tris or triethanolamine buffers at pH 8.5 could not be characterized by simple monophasic kinetics when the reaction was started with D-glyceraldehyde-3-P (Fig. 1). In all these experiments a lag period of about 5 sec was measured.

However, if the reaction was started with the enzyme, the time curve showed a simple monophasic character and the initial velocity was much higher.

In Fig. 2 the effect of D,L-glyceraldehyde-3-P and D-glyceraldehyde-3-P on the shape of the time curve are compared. The results indicate that the kinetics

depended on the conditions of preincubation of the enzyme, in particular on the presence of glyceraldehyde-3-P in the preincubation mixture. A lag period was also observed with the holo- and apoenzymes if the preincubation mixture did not contain glyceraldehyde-3-P (cf. Figs 1 and 2) whereas both D-and D,L-glyceraldehyde-3-P abolished the initial lag of NADH formation. In the presence of D,L-glyceraldehyde-3-P the activity was very low because of the inhibitory effect of L-glyceraldehyde 3-P (Keleti et al., 1973).

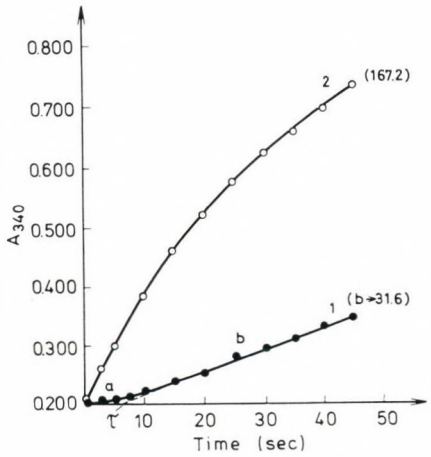

Fig. 1. Time course of the glyceraldehyde-3- phosphate dehydrogenase reaction. The measurements were carried out at 20 °C in an Opton PMQ II spectrophotometer (d = 10 mm) with a rapid mixing attachement. The buffer was 0.025 M Hepes, pH 8.5. The assay mixture contained 2.3×10^{-3} M NAD^+, 2×10^{-3} M D-glyceraldehyde-3-P, 10^{-2} M Na_2HPO_4 and 3 μg/ml holo-enzyme, if the reaction was started with glyceraldehyde-3-P (●) or 1.5 μg/ml holo-enzyme if the reaction was started with the enzyme (○). Values in parentheses indicate enzyme activity in IU calculated from phase b (curve 1) and from the initial rate data (curve 2). τ = time lag as obtained by the extrapolation of the steady-state slope

Further, we tested the effect of some L-α-amino acids, SH compounds and chelating agents on the shape of the kinetic curve. All of these was found to activate the enzyme and if applied in appropriate concentrations to change the complex kinetic curve into a simple monophasic one. Figure 3 illustrates the effect of alanine, added to the assay mixture together with NAD^+ and P_i, on the kinetics of glyceraldehyde-3-phosphate dehydrogenase reaction started by D-glyceraldehyde-3-P. When the reaction was started with the enzyme (not shown here) the activating effect was much less (125 – 136 % at maximum).

In a series of experiments we investigated the activating effect of a group of L-α-amino acids (Fig. 4), serine, glycine, alanine, arginine, phenylalanine, glutamic acid, and lysine exhibited similar activating patterns. Aspartic acid, even in concentrations as low as 1 mM, had a strong activating effect, and the effect of histidine had a maximum at 5 – 10 mM. It should be noted that glutamic

acid compared to aspartic acid, as well as histidine compared to arginine and lysine revealed different activating effects. D,L-α-amino acids are also strong activators (not shown here).

Figure 5 illustrates the activating effect of the SH compounds cysteine, 2-mercaptoethanol and dithiothreitol, and Fig. 6 that of the chelating agents EDTA, 8-hydroxyquinoline and o-phenantroline. Using the latter two classes of compounds maximum curves were obtained.

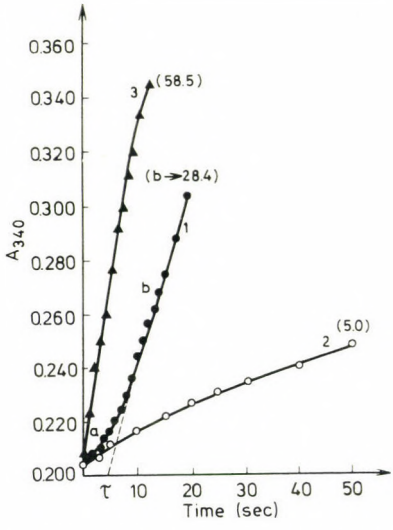

Fig. 2. Effect of glyceraldehyde-3-P, EDTA and glycine on the activity of glyceraldehyde-3-phosphate dehydrogenase. Solutions containg apo-enzyme, NAD^+, glyceraldehyde-3-P, and P_i in 0.025 M Hepes buffer, pH 8.5, were mixed in a Durrum stopped-flow apparatus. The formation of NADH was followed spectrophotometrically at 340 nm (d = 20 mm). The temperature was 20 °C. Values in parentheses indicate enzyme activity expressed in IU. The reaction mixture contained NAD^+ and P_i as indicated in Fig. 1, apo-enzyme (3 μg/ml), D-glyceraldehyde-3-P (2.7×10^{-3} M), D,L-glyceraldehyde-3-P (5.6×10^{-3} M) EDTA (10^{-3} M) and glycine (75×10^{-3} M). The experiments differ in the order of addition (compounds within brackets were in the same syringe). 1,(apo-enzyme + NAD^+ + P_i) + (D-glyceraldehyde-3-P); 2, (apoenzyme + D,L-glyceraldehyde-3-P) + (NAD^+ + P_i); 3, (apoenzyme + D-glyceraldehyde-3-P) + (NAD^+ + P_i) . τ = time lag

The structural feature is common for all activators used in the presence of groups containing N, S or 0 atoms on either side of two adjacent carbon atoms (Fig. 7).

In the molecule of dithiotreitol this structure is repeated twice and in the EDTA molecule 5 times. In the molecules of 8-hydroxyquinoline and o-phenantroline the distance between the carbon atoms is a little less than the bond between the adjacent atoms and no free rotation around this bond is possible. If we compare these compounds with glyceraldehyde-3-P or glyceric acid-1,3-diP, one can see

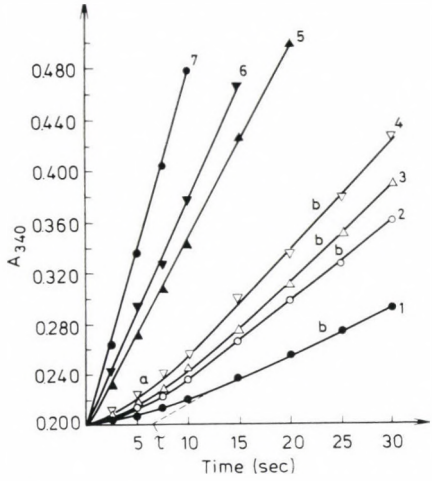

Fig. 3. Effect of L-α-alanine on the activity of glyceraldehyde-3-phosphate dehydrogenase. Conditions as in Fig. 1. Enzyme concentration: 3 μg/ml. The reaction was started with D-glyceraldehyde-3-P, 1, no addition; 2,3,4,5,6 and 7, alanine added at a concentration of 1,5,10,40, 100 and 150 mM respectively

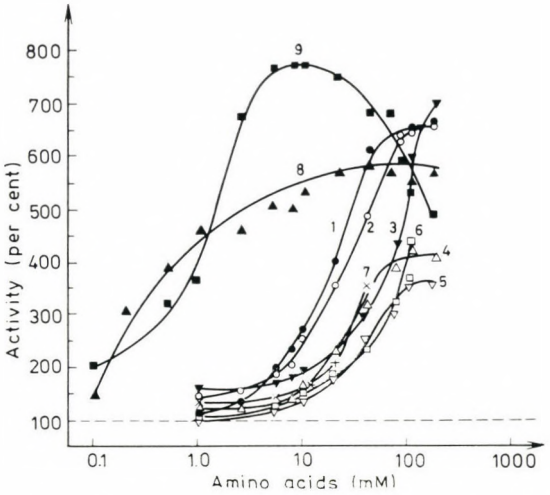

Fig. 4. Effect of the concentration of L-α-amino acids on enzyme activity. Conditions as in Fig. 1. Enzyme concentration: 3 μg/ml. The reactions were started with D-glyceraldehyde-3-P. Control, no addition (taken arbitrarily as 100%); 1, serine, 2, glycine; 3, alanine; 4, arginine; 5, lysine, 6, glutamic acid; 7, phenylalanine; 8, aspartic acid; 9, histidine added

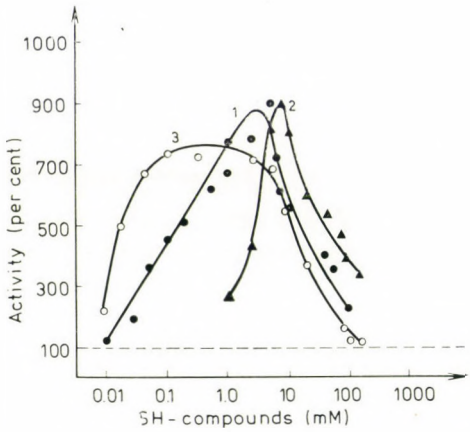

Fig. 5. Effect of the concentration of SH compounds on enzyme activity. Conditions as in Fig.1. Enzyme concentration: 3 μg/ml. The reactions were started with D-glyceraldehyde-3-P. Control, no addition (taken arbitrarily as 100%); 1, dithiotreitol; 2, 2-mercaptoethanol; 3, cysteine added

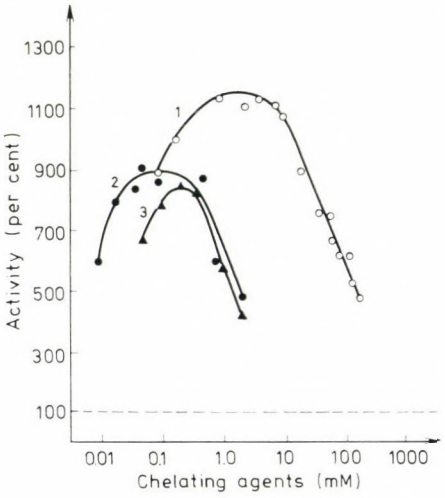

Fig. 6. Effect of the concentration of chelating agents on enzyme activity. Conditions as in Fig. 1. Enzyme concentration: 3 μg/ml. The reactions were started with D-glyceraldehyde-3-P. Control, no addition (taken arbitrarily as 100%); 1, EDTA, 2, o-phenantroline; 3, 8-hydroxyquinoline added

that they also contain two oxygen atoms at two adjacent carbon atoms. We assume that owing to the free electron pairs of N, S and O atoms these bi- or polyfunctional activators may act as proton acceptors when bound to the enzyme molecule.

Considering the activating effect of excess D-glyceraldehyde-3-P (Batke, Keleti, 1968), and the fact that the possibly common mechanism of activation by all compounds used might be related to the similarity of their structure to that

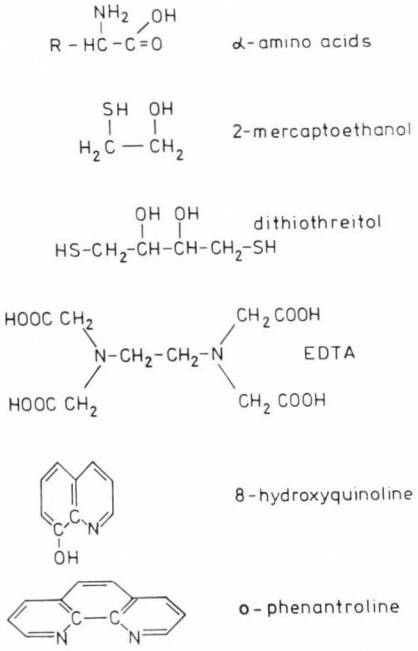

Fig. 7. Chemical structure of the effectors used

of glyceraldehyde-3-P, we carried out an experiment in which the concentration of D-glyceraldehyde-3-P was reduced and suitably chosen concentrations of glycine, EDTA and o-phenantroline were added (Fig. 8). In these experiments by replacing four fifths of the substrate by 0.1 mM o-phenantroline, 1 mM EDTA, or 100 mM glycine, nearly the same initial rate was obtained.

These findings are consistent with the claim of Nagradova (1965) that the higher the substrate concentration the lower EDTA concentration is needed to obtain the same reaction rate.

We suggest that both the excess of D-glyceraldehyde-3-P and the activators can bind to the regulatory center of the enzyme (Ovádi et al., 1972). This center may have at least 2 proton donor groups at a distance of 2 adjacent carbon atoms. Both the activators and D-glyceraldehyde-3-P bound to the regulatory center

can induce a similar conformational change in the glyceraldehyde-3-phosphate dehydrogenase molecule.

One may assume that the "activating effect" described is due to an acceleration by the effectors of the slow diol-aldehyde transformation which in some cases can be the rate limiting step of the glyceraldehyde-3-phosphate dehydrogenase reaction (Trentham et al., 1969). However, the rate constant of this conversion is $k = 0.06\ sec^{-1}$ (Trentham et al., 1969), whereas in our conditions the pseudo

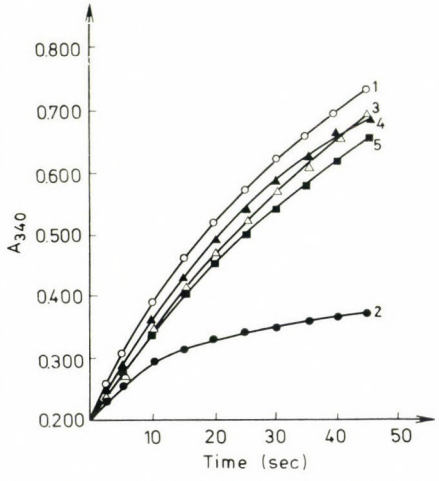

Fig. 8. Time-course of the reaction of glyceraldehyde-3-phosphate dehydrogenase with D-glyceraldehyde-3-P at different concentration. Conditions as in Fig. 1. Enzyme concentration: 1.5 μg/ml. The reactions were started with the enzyme, 1, D-glyceraldehyde-3-P (2×10^{-3} M), no addition; 2, D-glyceraldehyde-3-P (4×10^{-4} M) no addition; 3, D-glyceraldehyde-3-P (4×10^{-4} M) + EDTA (1×10^{-3} M); 4, D-glyceraldehyde-3-P (4×10^{-4} M) + glycine (10^{-1} M); 5, D-glyceraldehyde-3-P (4×10^{-4} M) + o-phenantroline (10^{-4} M)

first-order rate constant of the glyceraldehyde-3-phosphate dehydrogenase reaction (without any effectors, cf. Fig. 1, curve 2) is about 300 sec^{-1}. This fact definitely rules out any possible catalytic effect of amino acids, thiol compounds and chelating agents on the diol-aldehyde conversion of glyceraldehyde-3-P and similarly on hydrolysis of the imine of the aldehyde form with Tris ($k = 0.0002\ sec^{-1}$ in 0.05 M Tris, pH 8.5, cf. Ogilvie, Whitaker, 1976). However, at high (inhibitory) concentrations of thiol compounds the formation of thiazolidones as potential inhibitors of the enzyme cannot be excluded.

References

Batke, J., Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 385
Constantinides, S. M., Deal, W. C. (1969) J. Biol. Chem. *244* 5695
Cseke, E., Boross, L. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 385

Dimitrieva, L., Tomova, N., Dimova, O. (1974) Abstr. 9th FEBS Meeting, Budapest, p. 39., N s2a15
Elődi, P., Szörényi, E. T. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339
Horecker, B. L., Kornberg, A. (1948) J. Biol. Chem. *175* 385
Keleti, T. (1966) Acta Physiol. Acad. Sci. Hung. *29* 101
Keleti, T., Batke, J. (1965) Acta Physiol. Acad. Sci. Hung. *28* 195
Keleti, T., Telegdi, M. (1959) Acta Physiol. Acad. Sci. Hung. *16* 235
Keleti, T., Batke, J., Tro, T. Q. (1973) Acta Biol. Med. Germ. *31* 175
Nagradova, N. K. (1958) Biokhimiya *23* 511
Nagradova, N. K. (1965) Biokhimiya *30* 50
Ogilvie, J. W., Whitaker, S. C. (1976) Biochim. Biophys. Acta *445* 525
Oguchi, M., Meriwether, B. P., Park, J. H. (1973) J. Biol. Chem. *248* 5562
Ovádi, J., Telegdi, M., Batke, J., Keleti, T. (1971) Eur. J. Biochem. *22* 430
Ovádi, J., Nuridsány, M., Keleti, T. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. 7 133
Stancel, G. M., Deal, W. C. (1969) Biochem. Biophys. Res. Comm. *31* 398
Stancel, G. M., Deal, W. C. (1969a) Biochemistry *8* 4005
Szewczuk, A., Wolny, E., Wolny, M., Baranowski, T. (1961) Acta Biochim. Polon. *8* 201
Tomova, N., Setchenska, M., Krusteva, N., Christova, Y., Detchev, G. (1972) Z. Pflanzenphysiol. *67* 113
Tomova, N., Setchenska, M., Dimitrova, L., Dimova, O. (1973) Compt. Rend. Acad. Bulg. Sci. *27* 411
Tomova, N., Batke, J., Keleti, T. (1974) Abstr. 9th FEBS Meeting, Budapest. p. 40, N s2a16
Trentham, D. R., McMurray, C. H., Pogson, C. I. (1969) Biochem. J. *114* 19
Velick, S. F., Hayes, J. E., Harting, J. (1953) J. Biol. Chem. *203* 527

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Kinetic Studies on the Calcium-Dependent Potassium Transport in Human Red Blood Cells

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The kinetics of the Ca-dependent K transport across the human red cell membrane were studied under experimental conditions allowing the separation of this step from the previous membrane events. Net potassium and tracer potassium fluxes were measured and a self-consistent model was constructed for calculating the ionic permeability values. All the experimental results could be interpreted by means of a non-saturable neutral K selective channel. In net K efflux measurements the accompanying Cl movement was found to be rate-limiting, and the charge-carrying Cl permeability of the red cell membrane could be calculated.

Introduction

The membrane red cells incubated *in vitro* is known to become selectively permeable to K ions under various experimental conditions (e. g. high concentrations of NaF, iodoacetate + metabolic depletion, iodoacetate + inosine, triosereductone, propranolol or the Ca-ionophore A-23187, see for ref. Orringer, Parker, 1973; Riordan, Passow, 1973; Gárdos et al., 1975). In any of these cases the Na-permeability of the membrane remains unaffected and the phenomenon is highly Ca-dependent. The overall kinetics of this Ca-dependent K transport consist of at least four steps: 1. entry of Ca into the cells; 2. binding of Ca ions to specific membrane receptors; 3. formation of the K-transporting configuration of the membrane; 4. translocation of K ions (Lew, 1974; Szász, Gárdos, 1974). In the present work an attempt was made to study the fourth step separately and to construct a self-consistent model for all the observed characteristics of the K transport step.

Preliminary considerations

A) In order to separate the K transport step from the preceeding ones, kinetic measurements were carried out by using propranolol-treated red cells, in which ions were transported across the membrane at a maximum rate and efficacy (Szász et al., 1974; Gárdos et al. 1975).

B) To maintain electroneutrality, a net K transport has to be accompanied by a cotransport of anions and/or a countertransport of cations. The orders of

magnitude of ionic concentrations and permeabilities suggest that under our experimental conditions the only candidate for this role is chloride. Thus a net transport of K ions through the membrane can occur only with a simultaneous net transport of an equal amount of Cl ions. Attention should be paid to two peculiarities of this process.

1. Tracer exchange experiments on red cell membranes indicate a Cl permeability of about 10^{-4} cm/s (Tosteson, 1959). However, in strong contrast with this "exchange permeability" value, the charge-conducting "net permeability" of Cl ions appears to be about 10^{-8} cm/s both in transport experiments on valinomycin-treated cells (Hunter, 1971) and in membrane potential measurements (Lassen, 1972; Lassen et al., 1973). Whatever the origin of the discrepancy between the two values, the relatively low net Cl permeability might be a rate-limiting factor in Ca-dependent K transport (Glynn, Warner, 1972; Hoffman, Knauf, 1973; Gárdos et al., 1975).

2. The enormously high water permeability of the red cell membrane results in an osmometric behaviour of the cells: the cell volume is determined by the condition of identical extra- and intracellular osmotic pressures (practically, by the identical total concentrations of solutes). Beyond the necessity of taking this condition into account in the calculations, we faced a more disturbing fact. During a net KCl loss the cells shrank, and at a certain extent of cell shrinkage (roughly at a haemoglobin concentration of 65 per cent) the haemoglobin got "frozen" into a crystalline-like state sequestering the dissolved ions (Szász et al., 1974). At this point Ca-dependent K transport stopped in spite of the apparently non-zero driving force. Kinetic measurements were to be carried out in the region before this point was reached (under the conditions of our experiments in the first 20–25 minutes).

C) Three types of experimental set-up were used in parallel measurements:

(1) "K exchange": tracer flux (^{42}K; hereafter: K*) due to Ca-dependent K transport was measured at identical external and internal ionic compositions.

(2) "Net K efflux": increase in external K concentration due to Ca-dependent K transport was measured at physiological ionic composition (high internal – low external K concentrations).

(3) "K influx": K* was added to the medium and the change of internal activity was followed while Ca-dependent K* transport took place under physiological conditions.

"K exchange" measurements yielded K-permeability directly. The results obtained from "net K efflux" experiments allowed us to construct a model for determining net Cl-permeability. "K* influx" experiments served as a consistency check to the model, since permeability data obtained from the former two types of experiments could be used to simulate K* influx and the calculated and measured curves could be matched to prove or disprove the model.

Materials and methods

Chemicals: All chemicals were of reagent grade. Propranolol was a kind gift of V. Manninen.

Washed red cells of freshly drawn, heparinized human blood were used.

Na- and K concentrations in the medium were determined by flame-photometry using an EEL flame-photometer. Na- and K contents of the cells were measured in the TCA extract of cells washed three times with cold isosmotic choline-chloride.

^{42}K activity (specific activity = 0.159 Ci/g) was counted in a Beckman Biogamma scaler.

Haemoglobin was measured with the cyanomethaemoglobin method. Haematocrit was assessed by the microhaematocrit tube technique or by ^{125}I dilution.

Tracer influx was studied in suspension of a haematocrit of 20–30 per cent. The ingredients were mixed in an ice-cold water bath, then the suspension was transferred to the incubation temperature and shaken vigorously. Red cells of 0.5 ml samples were separated by rapid centrifugation (1 min, 10 000 g) through an ice-cold, 7 ml sucrose cushion (0.7 M sucrose in 0.16 M NaCl). The separated red cell sediment was rinsed thoroughly with 0.16 M NaCl and haemolysed with 1.0 ml of 0.1% saponin.

Tracer efflux from the K*-preloaded cells was followed in a suspension of a haematocrit of 2–3 per cent. Samples were centrifuged at 10 000 g for 1 minute and aliquots of the supernatants were counted.

Principles of kinetic calculations

The aim was to calculate the K- and Cl permeability values during Ca-dependent K transport and perform a consistency check for the model applied. We attempted to employ the simplest model yet providing a sufficient fit to the experimental data. To this end we made use of the following simplifying assumptions:

1. Permanent osmotic equilibrium between the cell interior and the external medium is ensured by an identical total concentration of K, Na, Cl, and haemoglobin. Possible changes in the osmotic coefficients were neglected.

2. Electroneutrality conditions concerning the transporting section of the membrane are fulfilled by taking into account K and Cl ions only as charges species.

3. The transport is quasi-stationary, that is, at any moment, the fluxes assume their stationary values corresponding to the momentary values of boundary conditions (internal and external concentrations).

4. Ionic fluxes through the membrane can be obtained by integrating the local Nernst-Planck flux equation under the following conditions:

(i) electroneutrality of flow (equality of K- and Cl fluxes);

(ii) local electroneutrality throughout the membrane (equality of intramembrane K- and Cl concentrations).

(iii) Donnan-like quasi-equilibrium distribution of K- and Cl ions between the membrane and the adjacent solutions.

The following notation will be used:

V volume (l);
n_A chemical quantity of species A (mmoles);
c_A molar concentration of species A (mmoles /l);
J_A net transport rate of species A (outward positive) (mmoles /s);
P_A permeability coefficient of species A (cm/s);
δ membrane thickness (cm);
Ω surface area of membrane (cm^2);

upper case indices:

I values in the cell interior;
II values in the external medium;
o initial values.

The extensive values (V, n_A, J_A) refer to 1 l red blood cell suspension. Concentrations in the cell interior are referred to cell water.

For each experimental run the initial values were determined and the time course of c_K^{II} and/or n_K^I was followed continuously. The fundamental equations of change are
the conservation of volume:

$$V^I + V^{II} = V^{I0} + V^{II0} = V = \text{constant};$$

the transport equations:

$$dn_A^I/dt = -dn_A^{II}/dt = -J_A, \ (A = K, K^*, Cl);$$

the impermeability conditions:

$$n_B^I = n_B^{I0}; n_B^{II} = n_B^{II0}, \ (B = Na, \text{haemoglobin});$$

the osmotic equilibrium conditions:

$$c_K^I + c_{Na}^I + c_{Cl}^I + c_{Hb}^I = c_K^{II} + c_{Na}^{II} + c_{Cl}^{II} \equiv 2c_0 = \text{constant};$$

the electroneutrality of flow:

$$J_K - J_{Cl} = O.$$

By elementary calculations this set of equations can be reduced to the

pair of differential equations

$$\frac{dc_K^{II}}{dt} = \frac{(c_0 - c_K^{II})^2}{c_0 n_{Na}^{II}} J_K$$

$$\frac{dn_{K*}^{I}}{dt} = - J_{K*}$$

(We assumed also $c_{Na}^{II} = O$, hence $c_K^{II} = c_{Cl}^{II} = c_0$.)

All other variables can be expressed in terms of the values of these two variables and the initial data.

The quasi-stationary transport rates J_K, J_{K*} are themselves the solutions of a pair of differential equations.

$$J_K = - \alpha\delta \frac{dc_K}{dx}$$

$$J_{K*} = - P_K \beta\delta \frac{c_{K*}}{c_K} \frac{dc_K}{dx} - P_K \delta \frac{dc_{K*}}{dx}$$

with

$$\alpha = 2\Omega \frac{P_{Cl} P_K}{P_{Cl} + P_K}$$

$$\beta = \frac{P_{Cl} - P_K}{P_{Cl} + P_K}$$

These equations can be obtained from the Nernst-Planck flux equations taking the aforementioned electroneutrality conditions

$$J_K - J_{Cl} = O\,,$$

$$c_K - c_{Cl} = O \text{ throughout the membrane}$$

into account. We have also the Donnan-like quasi-equilibrium boundary conditions:

at $x = O$ (internal face of the membrane):

$$c_K = c_{Cl} = (c_K^I c_{Cl}^I)^{1/2}\,,$$

$$c_{K*} = c_{K*}^I \left(\frac{c_{Cl}^I}{c_K^I}\right)^{1/2};$$

at $x = \delta$ (external face of the membrane):

$$c_K = c_{Cl} = (c_K^{II} c_{Cl}^{II})^{1/2}\,,$$

$$c_{K*} = c_{K*}^{II} \left(\frac{c_{Cl}^{II}}{c_K^{II}}\right)^{1/2}.$$

The solution is

$$J_K = \alpha\left[(c_K^I\, c_{Cl}^I)^{\frac{1}{2}} - (c_K^{II}\, c_{Cl}^{II})^{\frac{1}{2}}\right],$$

$$J_{K^*} = \alpha \frac{(c_K^I\, c_{Cl}^I)^{\frac{1}{2}} - (c_K^{II}\, c_{Cl}^{II})^{\frac{1}{2}}}{(c_K^I\, c_{Cl}^I)^{\frac{\beta+1}{2}} - (c_K^{II}\, c_{Cl}^{II})^{\frac{\beta+1}{2}}} \left[c_{K^*}^I (c_k^I)^{\frac{\beta-1}{2}} (c_{Cl}^I)^{\frac{\beta+1}{2}} - \right.$$

$$\left. - c_{K^*}^{II} (c_K^{II})^{\frac{\beta-1}{2}} (c_{Cl}^{II})^{\frac{\beta+1}{2}}\right]$$

It is worth noting that if $P_K \gg P_{Cl}$ then J_{K^*} can be expressed as

$$J_{K^*} = 2\Omega\, P_K \frac{(c_K^I\, c_{Cl}^I)^{\frac{1}{2}} - (c_K^{II}\, c_{Cl}^{II})^{\frac{1}{2}}}{\ln(c_K^I c_{Cl}^I) - \ln(c_K^{II} c_{Cl}^{II})} \left[\frac{c_{K^*}^I}{c_K^I} - \frac{c_{K^*}^{II}}{c_K^{II}}\right].$$

Moreover, if all the c' s except c_{K^*} are of the same value on both sides of the membrane ("exchange conditions") then

$$J_{K^*} = \Omega\, P_K (c_{K^*}^I - c_{K^*}^{II}).$$

Setting the expressions of the transport rates into the differential equations relating to c_K^{II} and $n_{K^*}^I$ and expressing all concentration variables in terms of c_K^{II}, $n_{K^*}^I$ and the initial data, we obtain a pair of differential equations of the form

$$dc_K^{II}/dt = \alpha f(c_K^{II};\ \text{initial data}),$$

$$dn_{K^*}^I/dt = g(c_K^{II}, n_{K^*}^I; P_K, P_{Cl};\ \text{initial data}).$$

The proper forms of these equations govern the "K exchange" as well the "net K efflux" and the "K influx", the only difference is in the initial conditions. The initial conditions and the permeability values given, the time course of c_K^{II} and $n_{K^*}^I$ as well as that of all other variables can be computed. On the other hand, from the initial data and the measured time course of c_K^{II} and $n_{K^*}^I$ the permeabilities P_K and P_{Cl} can be determined. We applied the following strategy:

(1) P_K was determined from the "K exchange" experiments directly.

(2) α was determined from the "net K efflux" experiments as

$$\alpha = \frac{1}{t} \int_{c_K^{II0}}^{c_K^{II}} \frac{dc_K^{II}}{f(c_K^{II};\ \text{initial data})}$$

and then

$$P_{Cl} = \frac{P_K\, \alpha}{2\Omega\, P_K - \alpha}$$

(3) The computed values of P_K and P_{Cl} were used to simulate the "K* influx" experiment and the calculated and measured curves were matched to investigate the self-consistency of our model.

Results

1. In the "K exchange" experiments the time-course of increase in external K* activity during K* efflux from K* preloaded and propranolol-treated red cells was followed at an identical external and internal ionic composition. As the rate of the movement of K was constant in a K concentration range of 5 – 160 mmoles/l (Gárdos et al., 1975), for kinetic measurements red cells with normal (high) K content, incubated in high-K medium were used.

In the absence of Ca ions the rate of K* efflux was very low: the half-time of the equilibration of K* was more than 3 hours, only slightly differing from that measured in the absence of propranolol. By adding Ca ions to the medium a rapid K* efflux developed instantaneously. The graph of its time course (followed at least through three half-times) was linear on a semilogarithmic plot (Fig. 1). As the low haematocrit made the K* reflux negligible, the rate constant could be used for a direct calculation of the K-permeability value. The P_K value obtained was 2.4 ($\pm$ 0.26) $\times 10^{-7}$ cm/s (n = 8).

2. "Net K efflux" measurements were made on fresh red cells containing a normal (high) amount of K ions and placed in an isosmotic high Na–low K medium. The time course of increase in external K concentration was followed after adding propranolol+Ca to the medium. In order to avoid a possible stimulation of the efflux rate by the initial rise of the external K concentration (Riordan, Passow, 1973; Gárdos et al., 1974), the initial medium K concentration was adjusted to 5 mmoles/l. The α values could be fitted to the measured changes in the

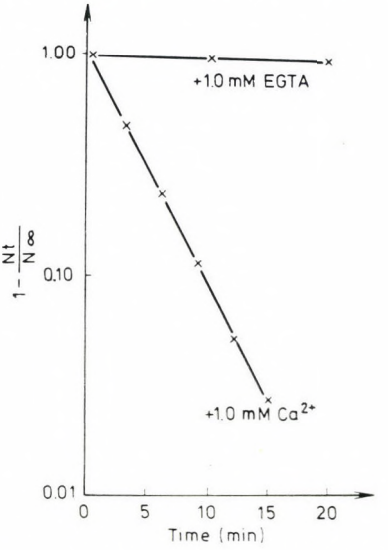

Fig. 1. ^{42}K efflux from human red cells in the presence of 0.5 mM propranolol. (Medium: 130 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4, 37 °C, haematocrit = 3%); N_t = = counts at moment t; N^∞ = counts as $t \rightarrow \infty$

medium K concentration. During the first 20–25 minutes of a given experiment α proved to be relatively constant (within deviations below 20 per cent). However, as a consequence of cell shrinkage (mentioned in Preliminary considerations) after this interval a significant decrease of α was observed. By computing the time-course of the net K efflux from the initial concentrations and the average α value, a smoothing of the experimental curve could be accomplished (Fig. 2a).

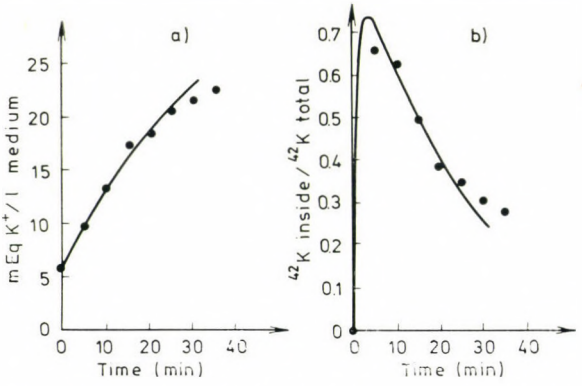

Fig. 2. Net K efflux (a) and ^{42}K influx (b) in red cells treated with 0.5 mM propranolol and 1mM Ca^{2+}. (Medium: 140 mM NaCl, 5 mM KCl, 15 mM Tris-HCl, pH 7.4, 37 °C, Haematocrit (at $t = 0$) = 30%). • measured values; — — — computed time-course

Estimation of P_{Cl} (net) of the red cell membrane was based on the average of the α values from 8 separate experiments. The value obtained for the net Cl permeability was $1.63\ (\pm 0.12) \times 10^{-8}$ cm/s.

3. "K* influx" was followed in the same experimental set-up as it was used for measuring net K efflux. During the net K loss a rapid K* uptake from the high Na -- ow K incubation medium could be observed first. After a transient tracer accumulation in the cells equilibration of K* took place. The initial concentrations and the parameters, P_K and P_{Cl} (net) being known in a given experimental system, a computer simulation of the K* influx could be carried out using the equations presented above (Fig. 2b).

Discussion

The main conclusions of the present work are as follows:

1. By completing the formation of the K-transporting membrane configuration with propranolol+Ca treatment, the kinetics of the K translocation step of Ca-dependent K transport could be studied separately. The rapid development of net efflux and K* movement as well as the unbroken exponential time-course of K exchange may indicate the validity of this statement.

2. A self-consistent model interpreting the results of all the three types of experiments could be constructed according to the following considerations:

(a) overall flux equations were obtained by integrating the local Nernst-Planck equations under the condition of local electroneutrality within the membrane (regarding K and Cl as the only permeable ions) and Donnan-like equilibrium conditions at the boundary interfaces;

(b) quasi-stationarity was assumed;

(c) isosmotic constraint was taken into account while formulating overall transport equations.

3. The estimated value of the permeability of K ions in Ca-dependent K transport was about an order of magnitude higher than the net Cl permeability. The calculated value of P_{Cl} (net) agrees well with the results obtained by others using different methods (Hunter, 1971; Lassen et al. 1973).

4. The good agreement between the simulated and measured time-course of K* influx indicates that our basic assumptions are most probably correct, i.e. the net efflux of K ions due to Ca-dependent K transport is accompanied by an equimolar efflux of Cl ions maintaining electroneutrality and is followed by a concomitant outflow of water preserving isosmoticity.

The mechanism of K movement in Ca-dependent K transport has been a central matter of discussion in numerous earlier papers. On the basis of the observed K activation and the characteristics of the K* influx, carrier-mediation of the process has been suggested (Ekman et al., 1969; Blum, Hoffman, 1972; Kregenow, Hoffman, 1972; Riordan, Passow, 1973). Others have found the arguments insufficient to prove this assumption (Glynn, Warner, 1972; Gárdos et al., 1975).

In the present paper we do not want to venture any unreliable hypothesis concerning the detailed mechanism of Ca-dependent K transport, all the more because kinetical arguments may be sufficient only to reject but not to accept molecular models. The only thing we can state is that the simplest realization of our kinetical model is a nonsaturable neutral K-selective channel the net K transport through which is accompanied by Cl movement, the latter being rate-limiting.

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References

Blum, R. M., Hoffman, J. F. (1972) J. Membrane Biol. *6* 315–328

Ekman, A., Manninen, V., Salminen, S. (1969) Acta Physiol. Scand. *75* 333–344

Gárdos, G., Szász, I., Sarkadi, B. (1975) in Biomembranes: Structure and Function (Gárdos, G., Szász, I., eds.) FEBS Proceedings, Vol. *35* pp. 167–180, North-Holland, Amsterdam

Glynn, I. M., Warner, A. E. (1972) Brit. J. Pharmacol. *44* 271–278

Hoffman, J. F., Knauf, P. A. (1973) in Erythrocytes, Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E., Wilmanns, W., eds.) pp. 66–70, Georg Thieme, Stuttgart

Hunter, M. J. (1971) J. Physiol. (London) *218* 49–50

Kregenow, P. M., Hoffman, J. F. (1972) J. Gen. Physiol. *60* 406—429
Lassen, U. V. (1972) in Oxygen Affinity and Red Cell Acid Base Status (Rorth, M., Astrup, P. eds.), pp. 291—304, Munksgaard, Copenhagen
Lassen, U. V., Pape, L., Vestergaard-Bogind, B. (1973) in Erythrocytes, Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E., Willmanns, W., eds.), pp. 33—36 Georg Thieme, Stuttgart
Lew, V. L. (1974) in Comparative Biochemistry and Physiology of Transport (Bolis, L., Bloch, K., Luria, S. E., Lynen, F., eds.). pp. 310—316, North-Holland, Amsterdam
Orringer, E. P., Parker J. C. (1973) in Progress in Haematology (Brown, E. B. ed.), Vol *8* pp. 1—23, Grune and Stratton, New York
Riordan, J. R., Passow, H. (1973) in Comparative Physiology, (Bolis, L., Schmidt-Nielsen, K., Maddrell, S. H. P., eds.) pp. 543—581, North-Holland, Amsterdam
Szász, I., Gárdos, G. (1974) FEBS Letters *44* 213—216
Szász, I., Sarkadi, B., Gárdos, G. (1974) Haematologia *8* 143—151
Tosteson, D. C. (1959) Acta Physiol. Scand. *46* 19—41

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Effect of a Biguanide (Buformin) on Carnitine Palmitoyltransferase Activity

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Buformin at a concentration of 1—2.5 mM stimulates solubilized carnitine palmitoyltransferase by reducing the inhibitory effect of palmitoyl-CoA. Neither buformin nor metformin inhibits the solubilized carnitine palmitoyltransferase.

Introduction

It is well established that the reaction catalyzed by CPT, (palmitoyl-CoA; L-carnitine O-palmitoyltransferase; EC 2.3.1.21) is a rate-limiting step in fatty acid oxidation and may be a key regulatory site (Fritz, 1968; Williamson et al., 1968; Lee, Fritz, 1971; McGarry et al., 1973; McGarry, Forster 1974; McGarry et al., 1975). Inhibitors of CPT (+)-octanoylcarnitine, (+)-decanoylcarnitine inhibit fatty acid oxidation and ketone body production and can restore the metabolic pattern in the liver from fasted animals (Williamson et al., 1968; McGarry et al., 1973; McGarry, Foster, 1974). One may suggest that other antiketotic and antidiabetic agents, such as biguanides, may act at this site. The finding that biguanides inhibit fatty acid oxidation (Muntoni et al., 1970; Corsini et al., 1974) supports this view. Although many effects of these antidiabetic drugs have been recorded, the primary target of their action is not yet known. In an attempt to ascertain the molecular site of attack, we tested whether buformin and metformin had any effect on CPT.

Materials and methods

The enzyme was prepared from bovine liver and was tested as described by Norum (1964) except that the enzyme unit was determined at 25 °C.

Enzyme activity was followed in three ways:

Radioactive exchange method to measure the CoA-dependent incorporation of L-(^{3}H) carnitine into palmitoylcarnitine (Bremer, Norum, 1967/a). Samples were dissolved in Bray's scintillation coctail.

Abbreviations used: CPT; carnitine palmitoyltransferase.

One-way reaction from left to right (see the equation in Results) to measure the L-(^{3}H)-carnitine liberated from (-)palmitoyl-(^{3}H)carnitine (Bremer, Norum, 1967/a). Samples were evaporated to dryness under an infrared lamp before dissolving them in Bray's solution. Radioactivity was measured in a Beckman LS-230 counter.

One-way reaction from right to left. In this newly developed system the SH group of released CoASH was detected by 4,4'-dithiobis-pyridine, a highly sensitive thiol reagent ($\varepsilon = 19.8$). In the presence of dithiobis-pyridine we could follow the reaction continuously using a Specord UV VIZ spectrophotometer. Dithiobispyridine inhibits neither CPT (our observation) nor carnitine acetyltransferase (Ramsay, Tubbs, 1975). In other cases ditiobispyridine was added after stopping the reaction (this time the reaction was going at pH 6.8).

(-)Palmitoyl-(^{3}H) carnitine was prepared by the above mentioned exchange reaction and palmitoyl-CoASH was prepared also enzymatically as described by Kornberg and Pricer (1953). DL-(Me^{3}H) carnitine was purchased from Radiochemicals (Amersham), Coenzyme A from Boehringer (Mannheim) 4,4'-dithiobispyridine from Fluka (Buchs). Buformin and metformin were gifts of Chinoin (Budapest) and Chemiewerk (Hamburg).

Results

First, the carnitine palmitoyltransferase reaction (-)palmitoylcarnitine + CoASH $\rightleftharpoons$ palmitoyl$-$SCoA + L-carnitine was studied by the exchange assay (Fig. 1). Buformin was found to enhance the incorporation of L-(^{3}H)-carnitine without shifting the equilibrium.

One-way reactions offer more detailed information. Figure 2 illustrates the results obtained by analyzing the one-way reaction from right to left. Substrate

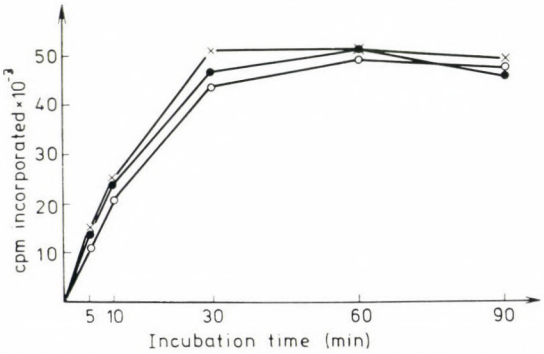

Fig. 1. Effect of buformin on the exchange reaction of carnitine palmitoyltransferase. The incubation mixture contained 240 μM (−) palmitoylcarnitine and L-carnitine, 100 μM CoA, 2 mM dithioerythritol, 250 000 cpm DL-(^{3}H)-carnitine, approximately 90 mU enzyme, 0.1 M Tris-HCl (pH 7.5) in a total volume of 1.0 ml at 30 °C. The figure is representative of four experiments. o−o no addition; ●−● 1 mM buformin; x−x 2.5 mM buformin

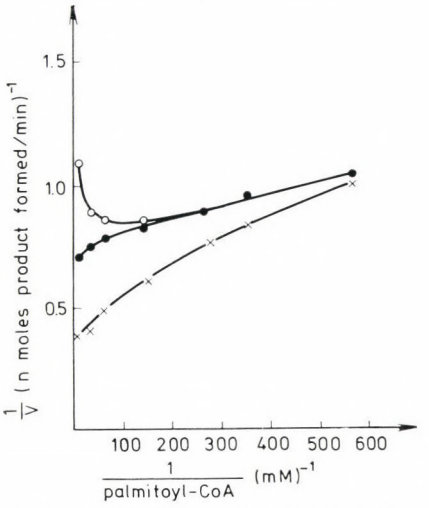

Fig. 2. The effect of buformin on the reaction (−) palmitoylcarnitine + CoASH ⇄ palmitoyl-CoA + L-carnitine from right to left. The incubation mixture contained 2 mM L-carnitine, 40 μM dithiobispyridine, 0.1 M Tris (pH 7.5), palmitoyl-CoA as indicated and approximately 1.5—2 mU enzyme in a total volume of 1.0 ml at 25 °C. The reaction was followed continuously. o—o no addition; ●—● 2.5 mM buformin; x—x calculated rate of a reaction with the enzyme saturated with L-carnitine (correction factor, $1 + \frac{K_m}{\text{L-carnitine}}$

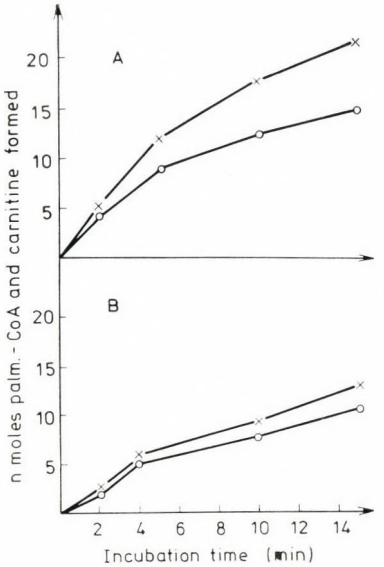

Fig. 3. The effect of buformin on the reaction (−) palmitoylcarnitine + CoASH ⇄ palmitoyl-CoA + L-carnitine from left to right. The incubation mixture contained 166 μM (−) palmitoyl-(^{3}H)carnitine (specific activity 800 cpm/nmol), 500 μM CoA, 2 mM dithioerythritol, 0.1 M Tris (pH 7.5) in a total volume of 0.6 ml at 30 °C. The figure is representative of five experiments. A; 7 mU enzyme, B; 3 mU enzyme. o—o no addition, x—x 2.5 mM buformin

inhibition, a well-known effect of palmitoyl-CoA (Bremer, Norum 1967/a) is clearly discernible and the figure shows a calculated curve free of substrate inhibition. Buformin activated the reaction partially by releasing the enzyme from the inhibitory effect of palmitoyl-CoA. We failed to demonstrate any inhibition, even if we reduced the concentration of the other substrate, L-carnitine. Adding the dithiobispyridine after the reaction had gone to completion we obtained the same results (data not shown).

Figure 3 shows the effect of buformin on the reaction from left to right. Since substrate inhibition operates in this direction as well the reaction becomes non-linear long before the equilibrium. Due to the increasing palmitoyl-CoA, concentration, an increasing stimulatory effect of buformin is observed. This finding is in good agreement with the data shown in Fig. 2. On changing the concentration of one or the other substrate, no further effect was observed. Metformin proved to be without effect in all the above assays.

Discussion

The stimulatory effect of buformin is due to the butyl group lending the compound a detergent character. Inhibitors of CPT are also known to have detergent properties. Inhibitors such as (-L)-palmitoylcarnitine, can also stimulate the enzyme at concentrations of palmitoyl-CoA, at which the inhibitory function of palmitoyl-CoA is prevailing (Bremer, Norum, 1967/a). The buformin concentration required seems to be too high for operating therepeutically. Yet, this finding raises the question whether buformin may affect other enzymes regulated by palmitoyl-CoA. Further, regarding the kinetics of CPT it is noteworthy that buformin can selectively counter-act the inhibitory function of palmitoyl-CoA without affecting its substrate function.

Almost all nontoxic effects of biguanides can be the consequence of an inhibition of fatty acid oxidation. Supposing the impairment is in the carnitine-system, one can suggest a better explanation for all the therepeutic effects of biguanides (Muntoni, 1974). In this light, it is important to emphasize the lack of inhibitory effect of these two biguanides on solubilized CPT.

Finally we stress that in this work the enzyme was in a solubilized state. Studies of a membrane-associated carnitine system may reveal other types of interaction with biguanides.

References

Bremer, J., Norum, K. R. (1967/a) J. Biol. Chem. *242* 1744

Bremer, J., Norum, K. R. (1967/b) J. Biol. Chem. *242* 1749

Corsini, G. U., Sirigu, S., Tagliamonto, P., Muntoni, S. (1974) Pharmacol. Res. Commun. *6* 253

Fritz, I. B. (1968) in Cellular compartmentation and control of fatty acid metabolism. Edited by Gran, F. C. Academic Press, New York

Kornberg, A., Pricer, W. E. (1953) J. Biol. Chem. *204* 329
Lee, L. P. K., Fritz, I. B. (1971) Can. J. Biochem *49* 599
McGarry, J. D., Meier, J. M., Foster, D. W. (1973) J. Biol. Chem. *248* 270
McGarry, J. D., Foster, D.W., (1974) J. Biol. Chem. *249* 7984
McGarry, J. D., Robles-Valdes, C., Foster, D. W. (1975) Proc. Nat. Acad. Sci. USA *72* 4385
Muntoni, S., Duce, M., Corsini, G. U. (1970) Life Sci. *9* 241
Muntoni, S. (1974) in Advances in Lipid Research, Academic Press, New York, *12*. p. 311
Norum, K. R. (1964) Biochim. Biophys. Acta *89* 95
Ramsay, R. R., Tubb, P. K. (1975) FEBS Letters *54* 21
Williamson, J. R., Bowning, E. T., Scholz, R., Kreisberg, R. A., Fitz, I. B. (1968) Diabetes *47* 194

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On the Enhanced Catalytic Activity of Papain towards Amide Substrates

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According to the scanty literature data papain (EC. 3.4.4.10) reacts with ester and corresponding amide substrates at a similar rate (Glazer, Smith, 1971) despite a considerable difference in the reactivities of the ester and amide bonds. An explanation for the similar rates may be an increased acylation rate of amides relative to that of esters owing to hydrogen bond formation between the amide group of an amide substrate and Asp-158 carbonyl oxygen as it is apparent from the three-dimensional structure of papain. This possibility was confirmed by comparing the second-order rate constants of acylation of papain with the ester and amide derivatives of N-benzoylglycine and O-benzoylglycolic acid. The rate enhancement with amides is not an equally important factor with all substrates of papain: the amides of N-acyl-L-phenylalanylglycine are hydrolyzed at a considerably lower rate than the corresponding esters. It is concluded from the above data that the binding mode is somewhat different with various substrates.

Introduction

The ratio of the rate constants of the reaction of papain with ester and corresponding amide substrates is remarkably low. In a detailed kinetic analysis Whitaker and Bender (1965) have shown that the second-order rate constants for the acylation of papain by Bz-L-arginine ethyl ester and Bz-L-arginine amide differ from one another only by a factor of about four. In the case of Bz-glycine ethyl ester (Sluyterman, 1964) and amide (Smith et al., 1958) the ratio of the rate constants of acylation is less than forty. On the other hand, in some other enzymatic hydrolyses (Neurath, Schwert, 1950; Bender, Kézdy, 1965) and in alkaline hydrolysis as well, esters were found to hydrolyze 3–4 orders of magnitude faster than amides. This high difference can be attributed to the fact that due to a higher resonance stabilization it is more difficult to cleave the $CO\text{-}NH_2$ than the CO-OR bond.

As for the unusual behaviour of papain, no explanation has been offered in the literature. Two questions may be raised in this respect. 1) What is the origin

Abbreviations: Nbs_2, 5,5'-dithio-bis(2-nitrobenzoic acid); Z, benzyloxycarbonyl; Bz, benzoyl.

of the relatively small difference in the rates of the papain-catalyzed hydrolyses of ester and amide substrates? 2) Is this small difference a general characteristic of papain catalysis or is it restricted to some substrates?

Materials and methods

Enzyme

Twice crystallized papain (Sigma Chemical Corporation) was purified on an agarose mercurial column prepared by the method of Sluyterman and Wijdenes (1970). The mercuripapain recovered from the column ($3-3.5\times10^{-4}$ M) was stored at 5 °C. No appreciable loss in activity was observed over a six months period. Appropriate amounts of mercuripapain were activated by the addition of an about 50-fold molar excess of 0.1 M cysteine adjusted to pH 6–7. After 30 minutes incubation at room temperature, the solution was gel-filtered through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer, pH 6.3, containing 1 mM EDTA. The protein fraction contained 0.95–1.0 mole of SH group/mole of papain as determined by titration with Nbs_2 (Ellman, 1959). Activated enzyme solutions could be stored in N_2 atmosphere at 5 °C with no more than 5% loss of activity per day.

The activity of papain was routinely tested with Z-glycine p-nitrophenyl ester as substrate (Kirsch, Igelström, 1966). This required much less enzyme than titration with Nbs_2. The hydrolysis was followed at 340 nm in 0.1 M acetate buffer, pH 5.5, containing 3.3% acetonitrile, 1 mM EDTA and 0.1 mM Z-glycine p-nitrophenyl ester at 25 °C. The reaction was started by the addition of the enzyme. Papain concentration was about 5×10^{-8} M in the cell. Under such conditions the reaction was zero order and proceeded at a high rate so that spontaneous hydrolysis of the substrate was negligible. The absorbance change was recorded by the 0.0–0.1 slide wire of a Varian Techtron 635 D spectrophotometer equipped with a constant temperature cell holder. The molarity of the active enzyme in the cell could be calculated by multiplying the reaction rate, expressed in change of absorbance units/min/cm, by a factor of 4.0×10^{-7}. This factor was obtained from titration of the freshly prepared papain with Nbs_2, at pH 8.2 where the equilibrium of the reaction is practically completely shifted towards the papain –5-mercapto-2-nitrobenzoate disulphide (cf. Little, Brocklehurst, 1972).

Substrates

N-Benzoylglycine ethyl ester was prepared according to Greenstein and Winitz (1961) by the thionyl chloride method from hippuric acid and anhydrous ethanol. After recrystallization from aqueous ethanol the pure ester melted at 58–59.5 °C. The literature (Lowe, Williams, 1965) records m.p. 59–60 °C.

N-Benzoylglycine amide was prepared by the addition of 2.5 ml cc. aqueous ammonium hydroxide to the solution of 2 g N-benzoylglycine ethyl ester in 2 ml

methanol. The crude product was recrystallized from water. M. p. 182–184 °C. The literature (Bergmann, Zervas, 1936) records m.p. 183–185 °C.

O-Benzoylglycolic acid ethyl ester was synthesized according to the procedure of Ringshaw and Smith (1964) for acetyl glycolic acid ethyl ester. Ethyl bromoacetate, benzoic acid and anhydrous Na_2CO_3 were refluxed in dry acetone for about 6 hrs. Acetone was removed *in vacuo*, the residue dissolved in water and extracted with ether. Ether extracts were dried over Na_2SO_4. After evaporation the residue was distilled. The fraction between 276–289°C was redistilled and a faintly yellowish oil was collected between 276–281 °C. The literature (Wislicenus, Andrieff, 1865) records b. p. 277–279 °C.

O-Benzoylglycolic acid amide was prepared after Riedel-de-Haen (1930). Equimolar amounts of chloroacetamide and sodium benzoate were melted together at 140 °C for 15 minutes. Recrystallization from water gave the pure product. M. p. 119–120 °C. The literature (Riedel-de-Haen, 1930) records m.p. 120–122 °C.

N-Benzyloxycarbonyl-L-phenylalanylglycine ethyl ester was synthesized as follows. To a stirred solution of Z-L-phenylalanine (9 g) in 120 ml tetrahydrofurane-acetonitrile (1 : 1) at −5 °C, glycine ethyl ester hydrochloride (4.4 g) dissolved in 12 ml dimethyl formamide and 4.4 ml triethylamine were added, followed by 6.5 g dicyclohexylcarbodiimide in 20 ml tetrahydrofurane. The suspension was stirred for 4 hrs between −5 and −10 °C, then allowed to stand overnight at + 5°C. Dicyclohexylurea was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and washed successively with water, 0.5 M HCl, water, saturated aqueous $NaHCO_3$, and water. The ethyl acetate layer was dried over $MgSO_4$. After evaporation the crude product was recrystallized from ethyl acetate-cyclohexane (1 : 5) mixture. The pure product proved to be homogeneous by thin layer chromatography. M. p. 108–109 °C. The literature (Fujino, Chitoshi, 1969) records m.p. 110 °C.

N-Benzyloxycarbonyl-L-phenylalanylglycine amide was obtained by the ammonolysis of the corresponding ester. 2 g ester was dissolved in 150 ml anhydrous methanol and gaseous ammonia was bubbled through the solution for 75 min at 0 °C. Then the gas-stream was stopped, the solution was allowed to warm up to room temperature and after sealing it was incubated for 30 minutes. Then methanol was removed *in vacuo* and the oily residue was precipitated by the addition of ether. The crude product was twice recrystallized from ethanol to give pure amide which proved to be homogeneous by thin layer chromatography. M.p. 129.5–131 °C. The literature (Fruton, Bergmann, 1942) records m.p. 134 °C.

N-Acetyl-L-phenylalanylglycine ethyl ester was prepared from the Z-derivative via catalytic hydration and acylation by p-nitrophenyl acetate at 0 °C in dimethylformamide. After evaporation *in vacuo*, the crude product was triturated with ether and then filtered. The material was recrystallized twice from ethyl acetate-cyclohexane (1 : 5) mixture. The pure product melted at 137–139 °C, and proved to be homogeneous by thin layer chromatography. The literature (Baumann et al., 1973) records m.p. 133–135 °C.

N-Acetyl-L-phenylalanylglycine amide was prepared from the corresponding ester by the method given above for the Z-derivative. After recrystallization from ethyl acetate the pure product proved to be homogeneous by thin layer chromatography. M.p. 179–181 °C. The literature (Baumann et al., 1973) records m.p. 183–184 °C.

Melting points were measured in a Tottoli type apparatus (Büchi, Switzerland) and are uncorrected. Thin-layer chromatograms were run on silica gel plates in the following solvent systems: ethyl acetate–pyridine–acetic acid–water (A) 240 : 20 : 6 : 11, (B) 60 : 20 : 6 : 11. Spots were detected with chlorine-tolidine.

Kinetics of hydrolyses

All reactions were performed at 25 °C. All solutions contained 1 mM EDTA and were made up with deionized water. Active papain concentration was checked during prolonged reactions and the loss of activity was within 5%.

Hydrolysis of esters was followed at 25 °C in a Radiometer pH-stat system consisting of a PHM 62 standard pH meter, a TTT 60 titrator, a REC 61 Servograph with a REA 160 titrigraph module, an ABU 12 autoburette with a 0.25 ml syringe and a TTA 31 titration assembly. The procedure in a typical run was as follows. The reaction mixture (4.5 ml) containing 0.15 M NaCl was placed in the thermostated cell. Nitrogen was bubbled through the solution for about 15 minutes. This did not cause any detectable decrease in the volume of the reaction mixture. When papain was added, the stream of nitrogen was directed over the solution. The pH of the reaction mixture was brought to 6.3 with the titrant from the autoburette or with more concentrated NaOH. The reaction was initiated by the addition of 50–100 μl stock solution of the substrate in ethanol. Ethanol concentration was always adjusted to 1.6–2% (v/v) in the reaction mixture. In the case of hydrolyses of dipeptide esters 2 to 5 mM NaOH, in all other cases 0.05–0.15 M NaOH was used as titrant.

Hydrolysis of amides was followed at 25 °C by determining the ammonia content with ninhydrin of aliquot samples from the reaction mixture as follows. The substrate was made up in 0.1 M phosphate buffer, pH 6.3, containing 1.6–2% (v/v) ethanol. The reaction was started by the addition of papain. Aliquots were withdrawn from the reaction mixture at appropriate times and were added to equal volumes of 2% ninhydrin solution containing 0.04% $SnCl_2 \cdot 2H_2O$ in a 1 : 3 mixture of 4 M acetate buffer, pH 5.5, and ethylenglycolmonomethylether (Spackman et al., 1958). Stannous chloride, by inactivating the enzyme, prevents further hydrolysis of the substrate in the samples. The samples were then heated in boiling water for 15 minutes and subsequently kept in an ice-water mixture for 3 minutes. If slight turbidity was observed, samples were centrifuged for 5 minutes at 4000 rpm. The absorption of the samples at 570 nm was measured. The blank solution was processed similarly except that the enzyme was omitted. A VSU-2 spectrophotometer was used for measuring the absorbance.

In the case of the hydrolysis of the dipeptide esters k_{cat} and K_m values were determined from Lineweaver-Burk plots. The k_{cat} value divided by K_m gives the second-order rate constant of acylation (Bender, Kézdy, 1965). In the case of the other substrates, where K_m values are higher, reactions were performed under pseudo first-order conditions. The pseudo first-order rate constant divided by the enzyme concentration also gives the second-order rate constant.

Results and discussion

1. Hydrogen bonding between substrates and Asp-158 of papain

Suggestions for the alignement of substrates at the active site of papain (Wolthers et al., 1970; Lowe, Yuthavong, 1971; Drenth et al., 1975) indicate that, in contrast to an ester substrate, an amide or peptide substrate is able to form a hydrogen bond, through its leaving -NH- function, to the carbonyl oxygen atom of Asp-158. This hydrogen bond might stabilize the leaving group in a position favourable for the proton transfer from His-159 thereby facilitating the reaction of papain with amide substrates. Another hydrogen bond can be formed between the same oxygen atom of Asp-158 and the α-amino group of an amino acid derivative substrate. To study the importance of these two hydrogen bonds in the catalysis, the ester and amide derivatives of N-benzoylglycine and O-benzoylglycolic acid were used. Each substrate displays a characteristic hydrogen bonding ability which is shown in Table 1.

Table 1

Second-order rate constants for the papain-catalyzed hydrolysis of O-benzoylglycolic acid and N-benzoylglycine derivatives at 25° C, pH 6.3

Substrate	Enzyme concentration	Range of initial substrate concentration	k	α-amino group--Asp-158 H-bond	leaving group-Asp-158 H-bond
	μM	mM	$M^{-1}s^{-1}$		
O-Bz-glycolic acid ethyl ester	24—38	1.3—2.3	7	impossible	impossible
O-Bz-glycolic acid amide	80—130	7—14	2	impossible	possible
N-Bz-glycine ethyl ester	1—3	1.3—7	250	possible	impossible
N-Bz-glycine amide	10—40	0.9—2.3	4	possible	possible

It is seen in Table 1 that the second-order rate constant for the hydrolysis of Bz-glycine ethyl ester is approximately 36 times as high as that of Bz-glycolic acid ethyl ester. However, the ratio of the rate constants is only about 2 in the

case of amide substrates. Accordingly, the rate-enchancing effect of the hydrogen bond between the α-amino group of substrate and Asp-158 seems to be decreased with amide substrates, where the hydrogen bond to the leaving group can also be formed.

It is also seen from Table 1 that the rate constant of the hydrolysis of Bz-glycolic acid ethyl ester is only about 3.5 times as high as that of the corresponding amide substrate. The same ratio is about one order of magnitude greater in the case of Bz-glycine derivatives. Apparently, the hydrogen bond between the leaving group and enzyme almost cancels the inherent stability difference between ester and amide bonds in the case of Bz-glycolic acid derivatives, where only one hydrogen bond to Asp-158 can be formed. With Bz-glycine derivatives the great difference between the rate constants shows that the rate enhancement is less if there is a possibility for the formation of another hydrogen bond with Asp-158. As the effect of either of the two hydrogen bonds is weaker in the presence of the other, it is reasonable to assume that there could be a competition between the leaving group and the α-amino group for a better hydrogen-bonding position to Asp-158.

2. Amidase activity of papain with substrates of different specificity

To decide whether the enhanced rate of amide hydrolysis is characteristic of papain catalysis itself or varies with substrates, we compared the rate constants for the papain-catalyzed hydrolysis of different ester-amide substrate pairs.

According to Schechter and Berger (1968) the cleavage of peptides containing a phenylalanine residue almost always takes place at the next but one peptide bond towards the C-terminus of the peptide. Therefore we choose the ethyl ester and amide derivatives of Z- and acetyl-L-phenylalanylglycine dipeptides as highly specific substrates. The second-order rate constants of acylation are shown in Table 2. It is seen that the substrates investigated display different k_{ester}/k_{amide} ratios. These remarkable differences (3.5–255) may be due to slightly different binding modes associated with various substrate pairs or with the amide and ester component of the same substrate pair. Further investigations are required to clarify how these binding modes depend upon the hydrogen bonds, the N-acyl group and the amino acid side chain of the substrate.

Our experimental results seem to support the suggestion that the enhanced reactivity of papain towards amide substrates is due to the formation of an additional hydrogen bond with amides as compared to ester substrates. This hydrogen bond may stabilize the leaving group of an amide substrate in a favourable position to accept a proton from the imidazolium ion. It is also apparent from our investigations that the extent of the rate enhancement by amides strongly depends upon the structure of the compound to be split.

Table 2

Rate constants for the papain-catalyzed hydrolysis of ethyl ester and amide substrates at 25°C, pH 6.3

Enzyme concentrations were as follows: dipeptide esters, 0.04 μM; dipeptide amides 0.4–0.6 μM. Substrate concentrations were as follows: Z-L-phenylalanylglycine ethyl ester, 0.005–0.05 mM (K_m = 0.02 mM); Z-L-phenylalanylglycine amide, 0.04–0.08 mM; acetyl-L-phenylalanylglycine ethyl ester, 0.01–0.10 mM (K_m = 0.04 mM); acetyl-L-phenylalanylglycine amide, 0.04–0.08 mM. For enzyme and substrate concentrations used in experiments with N-Bz-glycine and O-Bz-glycolic acid derivatives see Table 1. All reactions were performed in the presence of 2% alcohol

Acyl moiety of substrate	k_{ester}	k_{amide}	$\frac{k_{ester}}{k_{amide}}$
	$M^{-1}s^{-1}$	$M^{-1}s^{-1}$	
N-Z-L-phenylalanylglycine	510 000	2000	255
N-Acetyl-L-phenylalanylglycine	350 000[a]	1500	233
N-Bz-glycine	250[b]	4[c]	62
N-Bz-L-arginine[d]	1190	268	4.5
O-Bz-glycolic acid	7	2	3.5

[a] 170 000 $M^{-1}s^{-1}$ at 35 °C, pH 6.0; (Lowe, Yuthavong, 1971)
[b] 147 $M^{-1}s^{-1}$ as 40 °C, pH 6.0; (Sluyterman, 1964)
[c] 3.8 $M^{-1}s^{-1}$ at 38 °C, pH 6.0; (Smith et al., 1958)
[d] From Whitaker and Bender (1965), at 25 °C, pH 5.2

Note added in proof:

After the submission of the manuscript, we have learnt the paper of Drenth, J., Kalk, K. H. and Swen, H. M. (1976) Biochemistry *15* 3731 which shows by X-ray diffraction measurements the possibility of the formation of a hydrogen bond between the leaving amide group of the substrate and the backbone carbonyl group of Asp-158.

We gratefully acknowledge the generosity of Dr J. Drenth for providing us with the unpublished data on the refined co-ordinates of papain.

References

Baumann, W. K., Bizzozero, S. A., Dutler, H. (1973) Eur. J. Biochem. *39* 381
Bender, M. L., Kézdy, F. J. (1965) Ann. Rev. Biochem. *34* 49
Bergmann, M., Zervas, L. (1936) J. Biol. Chem. *113* 341
Drenth, J., Swen, H. M., Hoogenstraaten, W., Sluyterman, L. A. Ae. (1975) Proc. Kon. Ned. Akad. v. Wetenschap. C. *78* 104
Ellman, G. L. (1959) Arch. Biochem. Biophys. *82* 70
Fruton, J. S., Bergmann, M. (1942) J. Biol. Chem. *145* 253

Fujino, M., Chitoshi, H. (1969) Takeda Kenkyusho Nempo *28* 12
Glazer, A. N., Smith, E. L. (1971) in The Enzymes, 3rd. edn. Boyer, P. D. e., vol. 3. p. 501., Academic Press (1971) New York and London
Greenstein, J. P., Winitz, M. (1961) Chemistry of the Amino Acids vol. 2. p. 927. J. Wiley and Sons Inc., New York
Kirsch, J. F., Igelström, M. (1966) Biochemistry *5* 783
Little. G., Brocklehurst, K. (1972) Biochem. J. *128* 475
Lowe, G., Williams, A. (1965) Biochem. J. *96* 199
Lowe, G., Yuthavong, Y. (1971) Biochem. J. *124* 107
Neurath, H., Schwert, G. W. (1950) Chem. Rev. *46* 69
Riedel-de-Haen (1930) D.R.P. 555 931
Ringshaw, D. J., Smith, H. J. (1964) J. Chem. Soc. 1559
Schechter, I., Berger, A. (1968) Biochem. Biophys. Res. Commun. *32* 888
Sluyterman, L. A. Ae. (1964) Biochim. Biophys. Acta *85* 305
Sluyterman, L. A. Ae., Wijdenes, J. (1970) Biochim. Biophys. Acta *200* 593
Smith, E. L., Chavré, V. J., Parker, M. J. (1958) J. Biol. Chem. *230* 283
Spackman, D. H., Stein, W. H., Moore, S. (1958) Anal. Chem. *30* 1190
Whitaker, J. R., Bender, M. L. (1965) J. Am. Chem. Soc. *87* 2728
Wislicenus, J., Andrieff, V. (1865) Ann. *133* 284
Wolthers, B. G., Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M. (1970) Proc. Int. Symp. Struct. Funct. Relat. Proteolytic Enzymes p. 272 Munksgaard, Copenhagen

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Electron Spin Resonance Spectra of Chicken Liver and Hepatoma Tissue Embedded in Paraffin

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ESR spectra of chicken liver and hepatoma tissue embedded in paraffin display signals characteristic of free radicals and paramagnetic complexes. Owing to embedding the spectrum is altered as compared to that of the native tissue but remains characteristic of the respective tissues. The spectra of tissues embedded in paraffin allow no more than qualitative conclusions to be drawn concerning the spectra of the native tissues, even if appropriate controls are applied.

Introduction

Electron spin resonance spectra of biological tissues are usually measured in frozen samples (Swartz, 1972). The preparation, collection and storage for years in liquid nitrogen of frozen samples are expensive and trouble some tasks which are often inextricable in institutes of pathology. In such institutes, however, numerous samples of various pathological tissues embedded in paraffin are stored.

Therefore we thought it worth checking whether or not ESR spectra of embedded tissues would yield enough information for drawing conclusions concerning the ESR spectra of the respective tissues in their native state. For our experiments chicken liver and hepatoma tissues were chosen as experimental objects. The reason for this choice was that liver tissue had been known to give a relatively intensive spectrum as compared to other native tissues (Commoner, Ternberg, 1961) and, as reported by Elek et al. (1975) the spectrum of hepatoma tissue was characteristically different from that of the liver.

Methods

Samples frozen in liquid nitrogen (Elek et al., 1974) and embedded in paraffin were prepared from the livers of a dozen one-month-old Hunnia hybrid chickens. A similar amount of hepatoma was processed in the same way (Lapis et al., 1975) on the tenth day after passage. Embedding was carried out according to the routine histological procedure (increasing concentrations of ethanol, a methyl-benzoate bath and embedding in paraffin). In the course of the embedding

of some of the samples, methylbenzoate was replaced by butyl alcohol. The embedded tissue blocks were chaped to give rods measuring 2 cm in length 4 mm in diameter. As a control, a similar rod was prepared from that part of the paraffin block which contained no tissue. The spectra of rods and of the corresponding frozen samples were recorded in the same quartz Dewar flask. The only difference was that when recording the spectra of the frozen samples in the JES ME-3X spectrometer, the samples were cooled with liquid nitrogen, while the spectra of the paraffin rods were taken at room temperature.

Results

For a better comparison, the spectra are shown in the same figure (Fig. 1). The spectra presented are characteristic of the individual sample types. Paraffin may also exhibit paramagnetic absorbance owing to contaminations (a). This is especially striking in the case of bees wax, used as an additive in the course of embedding (b). After embedding the spectrum of hepatoma tissue, otherwise lacking characteristic features, became devoid of its slope between 0 and 3000 gauss, but was not changed in any other respect (c and d).

The spectrum of liver tissue differed from that of hepatoma also after embedding in paraffin – it contained more bands (cf. d and f). The slope observed in the frozen samples was absent also from liver samples embedded in paraffin. This is best seen in the 0–3000 gauss region. Another difference was that in the spectrum of liver tissue embedded in paraffin in the $g = 2.02 - 2.3$ region (2800–3200 gauss), the 300–400 gauss wide signal of Cu^{2+} was well discernible while it was absent from the spectrum of the frozen sample (g). This wide signal nearly completely covered the $g = 2.25$ signal of microsomal haem iron, which was clearly visible in the case of the frozen sample, but of which only the top of the peak would be guessed in the spectrum of the embedded sample. On the other hand, absorbance could be detected in the $g = 1.91$ region of haem iron and in the $g = 4.3$ region of Fe^{3+} (the latter was observed at approximately 1750 gauss). The spectrum of embedded liver tissue showed pronounced signals in the vicinity of metallic Mn. These were absent from the spectra of paraffin and bees wax (In spectrum *e* in Fig. 1, g values of the Mn signals shown for comparison are $g = 2.14$; 2.08; 2.03; 1.98; 1.92; 1.87 from left to right).

Figure 2 demonstrates that absorbance was nearly indiscernible at $g = 1.94$ (non-haem iron).

The signals of the embedded tissue samples were more intensive than those of the frozen samples (the former ones were recorded at half as large amplification). In every case the amplitude of the $g = 2$ signal of free radicals was found to be greater in the tissue embedded in paraffin than in the corresponding frozen sample. The signal of free radicals was absent from the spectra of the control paraffin rods. Spectra recorded after embedding in two different ways (methylbenzoate and butyl alcohol preparation) were essentially identical.

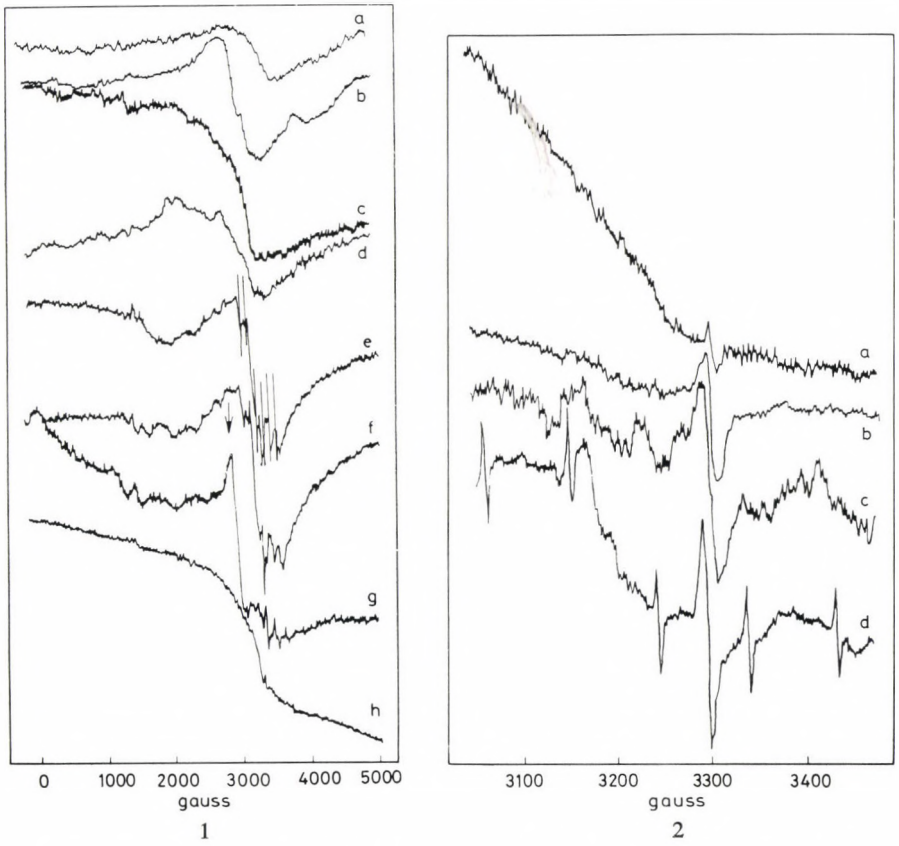

Fig. 1. X-banded ESR spectra in the 0—3000 gauss region of chicken liver and chicken hepatoma in frozen (native) state and embedded in paraffin. a) control paraffin rod; b) bees wax; c) frozen native hepatoma tissue; d) embedded hepatoma tissue; e) embedded liver tissue, together with a Mn control; f) same as e) without Mn control; g) frozen native liver tissue; h) distilled water at the temperature of liquid nitrogen. The arrow indicates the signal of microsomal haem iron, g = 2.26. The spectra were recorded under identical conditions. Microwave power: 10 mW, modulation: 12.5 gauss; time constant (τ): 1 sec; recording time (t): 5 minutes; amplification: a, b, d, e, f (room temperature) 500-fold; c, g and h, in liquid nitrogen 1000-fold

Fig. 2. X-banded ESR spectra in the 3100—3400 gauss region of chicken liver and hepatoma tissue in frozen state and embedded in paraffin. a) frozen hepatoma tissue; b) hepatoma embedded in paraffin; c) frozen chicken liver; d) chicken liver embedded in praffin, together with a Mn control. The Mn lines shown (from left to right) are as follows: g = 2.14, 2.08; 2.03; 1.98; 1.92. The spectra were recorded under identical conditions. Microwave power: 10 mW, modulation: 12.5 gauss; time constant (τ): 1 sec; recording time (t): 5 minutes; amplification: a) and c) in liquid nitrogen, 2000-fold b) and d), at room temperature, 1000-fold

Discussion

Tissues embedded in paraffin display paramagnetic absorbance which may come from the original paramagnetic substances in the native tissue but also from other compounds contaminating the tissue during embedding. However, the two tissues studied, possessing different ESR spectra in native state, showed characteristic differences after embedding as well. This suggest that – at least to some extent – the spectrum is characteristic of the given tissue also after embedding in paraffin.

After embedding the spectrum is changed as compared to that of the native tissue. The wide wave observed with frozen samples in the $g = 0-3000$ gauss region (which is often seen in spectra published in the literature, e.g. in the Figures presented by Chetverikov and Vanin (1968) and Vanin et al. (1968) dissappears after embedding. This slope is seen in spectra recorded at the temperature of liquid nitrogen and can be attributed to oxygen absorption (see h, in Fig. 1, the spectrum of distilled water.) Thus, the disappearance of the slope is due to differences between the conditions of measurement and not those between the samples.

After embedding a wide signal appears between 2800 and 3200 gauss which comes from copper (Sands, Beinert, 1960). This signal is well-known from isolated mitochondrial preparations and indicates the oxidized state of the sample (Bäckström et al., 1970). Its appearance in the embedded tissue, however, may also be due to the presence of bees wax, the spectrum of the latter substance also shows absorption in the same region. Therefore the spectra of embedded tissues can be evaluated only if they are compared to the spectra of control samples prepared from parts of the paraffin blocks containing no tissue. The spectra of embedded liver tissue show several bands in the vicinity of Mn signals (between 2970 and 3470 gauss), which may be attributed only to the tissue as they are absent from the corresponding controls.

It is difficult to make a quantitative comparison between the spectra of frozen and embedded samples. The main reason for this is that the tissue shrinks in the course of dehydration and embedding. The spectra of samples embedded in paraffin are positively more intensive than those of frozen samples. This may be due not only to shrinkage but also to the dielectric constant which is more favourable than that of ice. In the embedded samples the amplitude of the $g = 2$ signal of free radicals was significantly larger than that of other signals. As no absorbance occurs in paraffin in that region, it must be attributed to the tissue; however, the relative increase in amplitude may indicate an increase in the concentration of free radicals in the course of embedding. Similar changes in the concentration of radicals have been observed also after freeze-drying in an athmosphere containing oxygen (air) (Truby, Goldzieher, 1958).

Thus, in the course of fixation and embedding paramagnetic substances may be washed off and also get into the tissues. The oxidized or reduced state of the tissues is changed and this probably leads to an increased concentration of free

radicals. Such ESR spectra may be characteristic of certain tissues, still they do not reveal the native state of paramagnetic substances. What reasons are offered, then, for the investigation of such samples?

The main reason is that there are some metal complexes (Mn) which do not leave the tissue during embedding, neither is their g value changed to an extent to make identification impossible. Therefore we assume that evaluation of the spectra of embedded samples may be considered as a complementary test besides the study of fresh clinical material, especially when preliminary measurements are to be made to determine the frequency of the occurrence of certain paramagnetic complexes in pathological material and to decide if ESR spectroscopy is worth applying in their detailed investigation.

References

Bäckström, D., Norling, B., Ehrenberg, A., Ernster, L. (1970) Biochim. Biophys. Acta *197* 108

Chetverikov, A. G., Vanin, A. F. (1968) Biofizika (Moscow) *13* 255

Commoner, B., Ternberg, I. L. (1961) Proc. N.A.S. *47* 1374

Elek, G., Lapis, K., Rockenbauer, A., Tibay, T. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. *10* 181

Elek, G., Turcsányi, B., Holland, R., Ladányi, L. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 15

Lapis, K., Beard, D., Beard, J. W. (1975) Cancer Res. *35* 132

Sands, R. H., Beinert, H. (1960) Biochem. Biophys. Res. Comm. *3* 47

Swartz, H. M. (1972) Cells and Tissues. In: Biological Application of Electron Spin Resonance. Ed: H. M. Swartz, I. R. Bolton, D. C. Borg. Wiley-Interscience, New York—London, Chapter 4. p. 155

Truby, F. K., Goldzieher, J. W. (1958) Nature *182* 1371

Vanin, A. F., Chetverikov, A. G., Blumenfeld, L. A. (1968) Biofizika (Moscow) *13* 66

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Studies on Heart Isophosphorylases by Means of Immunotitration

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Anti-phosphorylase produced in cocks against phosphorylase from rabbit skeletal muscle inhibits phosphorylases isolated from mammalian heart and skeletal muscle to different degrees. On the basis of the differential inhibition observed an immunotitration method was developed to determine isophosphorylases in crude heart extracts. It was found that the ratio of isophosphorylases is different for every mammalian species investigated and characteristic for the given species.

Introduction

Phosphorylase (E. C. 2.4.1.1), the rate-limiting enzyme of glycogenolysis has been found to be a mixture of isoenzymes in the heart of several mammalian species. From this mixture, three isoenzymes could be isolated by chromatographic and electrophoretic methods (Yunis et al., 1962; Davis et al., 1967; Hanabusa, Kohno, 1969; Will et al., 1970; Vereb, Csornai, 1970). One isoenzyme proved to be similar to the phosphorylase of skeletal muscle (Yunis et al., 1962), the other was reported to be specific for heart, and the third to be a hybrid of the two previous types (Davis et al., 1967). Isophosphorylases from heart differ not only in chromatographic and electrophoretic behaviour but also in the kinetics of the catalyzed reaction (Hanabusa, Kohno, 1969; Will et al., 1970; Schlieselfeld et al., 1970; Vereb, Bot, 1972). However, the most significant differences have been found in their immunological properties.

The determination of the quantity and ratio of isophosphorylases is rendered possible by the fact that the antibody produced against skeletal-type phosphorylase not only precipitates the homologous antigen but also inhibits its activity. The inhibition is proportional to the quantity of antibody added (Yunis et al., 1962; Jókay, 1963). Thus, in addition to quantitative precipitation, inhibition of enzyme activity can also be used for measuring the effect of anti-phosphorylase on different isophosphorylases. It has been reported by Yunis et al. (1962) that anti-phosphorylase produced against rabbit skeletal muscle phosphorylase inhibits one of the heart isoenzymes (the so-called P_1 phosphorylase) and the skeletal-type enzyme to the same degree. The antibody reacts to a much lesser extent with the other isophosphorylase from heart (heart-type, P_3 phosphorylase)

with respect to both precipitation and inhibition of enzyme activity (Schlieselfeld et al., 1970).

On the basis of these large differences in the inhibition of different isoenzymes it was possible to work out an immunotitration method for the simultaneous determination of isophosphorylases in the same mixture. This method proved to be suitable for the determination of the ratio of skeletal-type and heart-type isoenzymes in the heart of different mammalian species. This ratio was found to be different and characteristic for every species studied.

Materials and methods

Preparation of phosphorylase b from rabbit skeletal muscle

The preparation of phosphorylase *b* from rabbit skeletal muscle was carried out as described by Fischer and Krebs (1958). The specific activity of the enzyme was 50 U/mg protein according to the phosphorylase test used in our laboratory. Protein was determined by the biuret method of Gornall et al. (1949).

Preparation of pig heart phosphorylase and separation of the isoenzymes

Phosphorylase *b* from pig heart was prepared according to the procedure developed in our laboratory (Vereb, Bot, 1972). Isoenzymes were separated by chromatography on DEAE- cellulose according to the method of Yunis et al. (1962), as modified by Vereb and Bot (1972).

The specific activities of P_1 and P_3 isophosphorylases obtained in this way were in the range of 8–11 U/mg protein.

Preparation of tissue extracts for antibody titration

Hearts and femoral muscles of rabbits, guinea pigs and rats were dissected and frozen in liquid air. Pig, bovine and sheep hearts were purchased from a slaughter-house and were frozen, tissues were homogenized in four volumes of a buffer solution containing 20 mM glycerophosphate + 1 mM EDTA + 10 mM mercaptoethanol (pH 6.8). The homogenate was centrifuged at 5000 g for 20 minutes at +5°C. The tissue extracts contained phosphorylase in the *b* from.

Measurement of phosphorylase activity

Phosphorylase activity was measured by the method of Illingworth and Cori (1953) based on glycogen synthesis. The reaction was carried out at 30 °C in a mixture containing 16 mM glucose-1-phosphate, 1% glycogen, 1 mM AMP

(pH 6.8) and an amount of enzyme which was capable of converting not more than 20% of the glucose-1-phosphate present during the incubation time. The reaction was stopped by the addition of TCA. After the elimination of proteins, inorganic phosphate was determined according to Taussky and Shorr (1953). One enzyme unit is defined as the amount of phosphorylase which converts 1 μmole of substrate in 1 minute.

Preparation of anti-phosphorylase

Cocks were immunized with phosphorylase *b* from rabbit skeletal muscle according to Jókay and Tóth (1966). 10 mg of the enzyme were injected subcutaneously at different places. The injection was repeated twice at weekly intervals. The enzyme dissolved in 0.5 ml of 0.01 M Tris-HCl buffer (pH 6.8) containing 0.15 M NaCl was supplemented with 0.5 ml of complete Freund adjuvant (Difco). After 6 weeks a booster injection (5 mg enzyme dissolved in the same solution, without adjuvant) was given.

Antibody titres were determined daily in blood samples taken from the crest of the animals. 7 to 8 days after the booster injection, at the time of maximal titre, the animals were bled. Equal volumes of saturated $(NH_4)_2SO_4$ solution were added to the sera obtained (approximately 12 ml from one animal). The globulin fraction precipitated in this way and containing anti-phosphorylase was spun down, dissolved in 6 ml of 0.15 M NaCl + 0.01 M Tris-HCl buffer (pH 6.8) and dialyzed against the same solution. The immunoglobulin solution was stored either frozen or in freeze-dried state. The anti-phosphorylase content of the immunoglobulin fraction was determined on the basis of enzyme inhibition. (According to the linear part of the titration curve shown in Fig. 1, 1 ml of the anti-phosphorylase preparation inhibits about 90 units of phosphorylase *b* from pig skeletal muscle.)

Determination of anti-phosphorylase by inhibition of phosphorylase activity

In order to quantitate the degree of inhibition, 80–100 mU of phosphorylase were incubated in the presence of 20 mM glycerophosphate + 1 mM EDTA + + 10 mM mercaptoethanol (pH 6.8) with diluted (1 : 30) control serum from untreated cocks and various amounts of anti-phosphorylase. The mixture (final volume: 0.2 ml) was kept at 30 °C for 10 minutes, then it was supplemented with an equal volume (0.2 ml) of substrate solution (2% glycogen, 32 mM glucose-1-phosphate, 2 mM AMP, pH 6.8) and the remaining enzyme activity was determined as described above.

Since the serum of untreated cocks increases the activity of crystalline phosphorylase, results were corrected for this effect. Substracting the activity measured in the presence of both anti-phosphorylase and control serum from those measured in tests containing control serum only, values characteristic of the inhibiting effect of anti-phosphorylase were obtained.

Results

The effect of antibodies produced against rabbit skeletal-type phosphorylase on the activity of P_1 and P_3 isophosphorylases from pig heart was studied. Figure 1 shows the inhibition of these two heart isoenzymes and of phosphorylase from pig skeletal muscle as a function of antibody concentration.

It is apparent that the P_1 isoenzyme from heart is inhibited in a similar way as phosphorylase from pig skeletal muscle is. Inhibition is linear up to 75%. To get a further increase in inhibition, relatively greater amounts of antibody are required. The identical inhibition of both P_1 isophosphorylase from heart and the skeletal type phosphorylase suggests that the two phosphorylases have identical antigen properties.

Antibodies against skeletal-type phosphorylase inhibit the P_3 fraction of pig heart only to a small extent. This means that, regarding antigen properties, the P_3 fraction of heart phosphorylases differs significantly from the skeletal-type enzyme; that is why the P_3 fraction is termed heart-specific.

It is this difference between the immunological properties of the two isophosphorylases from heart that makes their simultaneous determination possible. In order to work out a method based on differential inhibition, however, it was necessary to investigate the behaviour of the two isoenzymes when present simultaneously and to compare it with that of the chromatographically separated fractions. For this reason we studied the activity and inhibition by antiphosphorylase of enzyme mixtures containing the P_1 and P_3 fractions in different ratios. The results are demonstrated in Fig. 2.

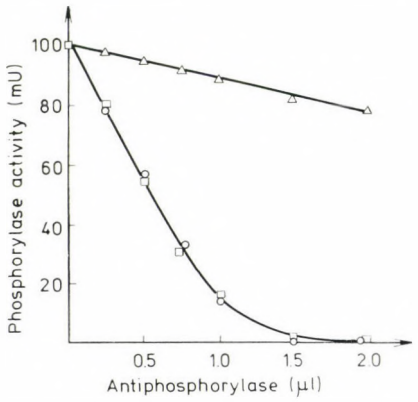

Fig. 1. Effect of antiphosphorylase produced against rabbit skeletal phosphorylase on P_1 and P_3 phosphorylases from pig heart. P_1 and P_3 isophosphorylases from pig heart were prepared by DEAE-cellulose chromatography; the extract containing skeletal-type phosphorylase from pig was prepared as described in Materials and Methods. Reaction mixtures for inhibition studies contained 100 mU of phosphorylase in every case. Abscissa: amount of antiphosphorylase (μl); ordinate: enzyme activity (mU) remaining after incubation with antibody. o——o, P_1 isophosphorylase; △——△, P_3 isophosphorylase; □——□, skeletal phosphorylase

It is shown that P_3 (heart-type) isophosphorylase alone is only slightly inhibited by antiphosphorylase produced against the skeletal-type enzyme. Upon adding increasing quantities of P_1 isophosphorylase, an increasing level of inhibition is achieved by the same amount of antibody. The length of the steep section of the curve representing inhibition versus antibody level depends on the quantity of the P_1 isoenzyme. From the intersection of the steep and flat parts of the inhibition curve the amount of P_3 isophosphorylase can be calculated, regardless of the quantity of P_1 isoenzyme present. Similar results were obtained

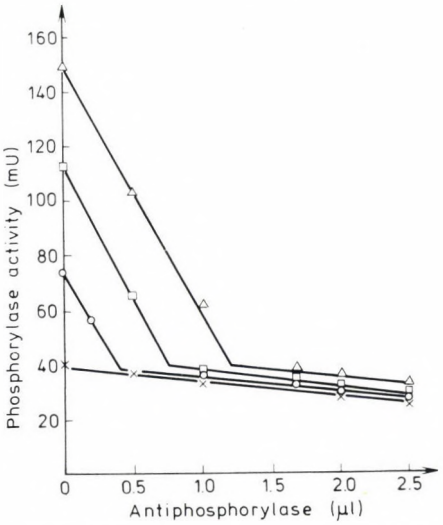

Fig. 2. Inhibition of mixtures of isophosphorylases from heart by antiphosphorylase against the skeletal-type isoenzyme. Mixtures containing 40 mU of P_3 isophosphorylase and varying amounts of P_1 isophosphorylases were incubated with increasing amounts of antiphosphorylase. The remaining activity was measured as described in Materials and Methods. x——x, 40 mU of P_3; o——o, 40 mU of P_3 + 35 mU of P_1; □——□, 40 mU of P_3 + 75 mU of P_1; △——△, 40 mU of P_3 + 110 mU of P_1

when the amount of P_3 isophosphorylase was varied and that of the P_1 isoenzyme kept constant.

The method worked out with purified enzymes can also be used for determining isophosphorylases in *crude* heart extracts. Immunotitration curves of heart extracts from different mammalian species are shown in Fig. 3.

It can be seen that the amounts of P_1 (skeletal-type) and P_3 (heart-type) isophosphorylases display a significant variation in the heart of different mammals. The isoenzyme ratios calculated from the points of intersections of the titration curves are summarized in Table 1.

It is apparent from the Table that the P_3 (heart-type) isophosphorylase is highly abundant in pig heart while it is present only at lower levels in rabbit,

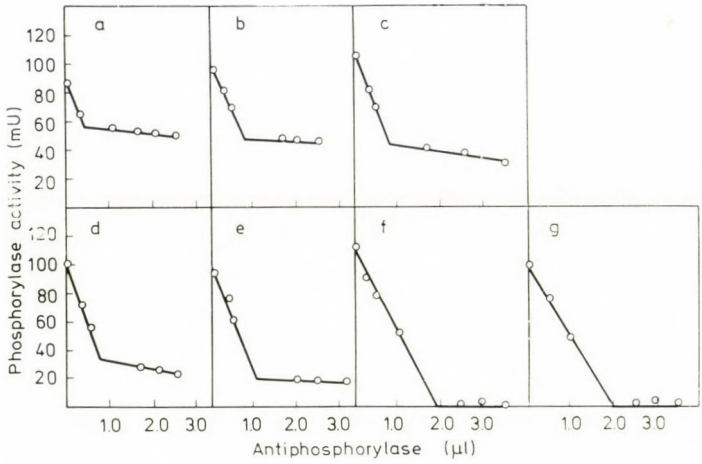

Fig. 3. Inhibition of phosphorylases in mammalian heart extracts by antiphosphorylase. Tissue extracts were prepared and measurements were carried out as described in Materials and Methods. The ordinate shows the remaining activity after incubation with various amounts of antibody. *a:* pig; *b:* bovine; *c:* human; *d:* rabbit; *e:* sheep; *f:* guinea pig; *g:* rat

Table 1

Percentage distribution of isophosphorylases in the heart of different mammalian species

Tissue extracts for immunotitration were prepared and immunotitration was carried out as described in Materials and Methods as well as in the legends to Fig. 3. The values represent the averages of 5 parallel determinations from different animals

Species	Heart-Type (P_3) Isophosphorylase %	Skeletal-Type (P_1) Isophosphorylase %
Pig	62±3	38±3
Bovine	50±5	50±5
Human	43±5	57±5
Rabbit	35±5	65±5
Sheep	22±3	78±3
Guinea pig	0	100
Rat	0	100

± Standard deviation

bovine and sheep heart. Guinea pig and rat hearts contain exclusively the P_1 (skeletal-type) isophosphorylase, while in human heart the P_3 (heart-type) isoenzyme makes up approximately 43% of the total phosphorylase content.

Discussion

Isophosphorylases from the heart of different animal species were first studied in a gel electrophoretic system by Davis et al. (1967). In some cases the isoenzyme compositions determined by gel electrophoresis and by immunological methods were in good agreement, e.g. only the skeletal-type isophosphorylase could be demonstrated in guinea pig and rat heart by both methods. In the case of bovine heart, however, the two methods yielded different results: gel electrophoresis revealed one isoenzyme while immunotitration indicated the presence of two isophosphorylases.

The immunological relationship of heart and skeletal-type phosphorylases was first studied by Henion and Sutherland (1957) who investigated the inhibitory effect of antiphosphorylases produced against heart and liver phosporylases of dog. These authors found no significant difference between the immunological properties of the two enzymes. This might be due to the fact that the heart phosphorylase used for immunization was a mixture of isoenzymes which they failed to fractionate chromatographically.

The immunological identity of chromatographically purified P_1 with skeletal type isophosphorylase from rabbit was shown by Yunis et al. (1962). In our experience, antiphosphorylase produced against rabbit skeletal phosphorylase inhibits the P_1 isoenzyme from the heart of every mammalian species. This fact is in good agreement with the results concerning the class-specificity of skeletal-type phosphorylase (Jókay et al. 1958). On this ground the ratio of P_1 and P_3 isophosphorylases could be determined in the heart of several mammalian species.

As judged from its immunological and electrophoretic behaviour (Schlieselfeld, 1973; Davis et al., 1964), the heart-specific phosphorylase resembles phosphorylase prepared from smooth muscle.

It is of interest to note that there is no such section in the titration curve of heart extracts with antiserum that might be attributed to the inhibition of P_2 (hybrid type) isophosphorylase. It is known that the hybridization of P_1 and P_3 isoenzymes results in a reversible equilibrium (Davis et al., 1967; Hanabusa and Kohno, 1969). Consequently, the dissociation of the P_2 hybrid to P_1 and P_3 isoenzymes might take place during immunotitration so that no P_2 hybrid can be observed. It is also possible, however, that the P_2 fraction is only an artefact formed during the purification process; fresh heart extracts may well contain only the P_1 and P_3 isoenzymes.

At present the significance of the differences between the isoenzyme ratios observed in different species is not known. In our experience this ratio is characteristic of every species investigated and is probably genetically determined.

The physiological significance of the occurrence of P_1 and P_3 isoenzymes lies probably in their different allosteric sensitivity. Heart-type phosphorylase *b* possesses 5 to 10 times higher affinity to AMP than the skeletal-type enzyme does. Accordingly, the heart-type phosphorylase can catalyze glycogenolysis more efficiently than the skeletal-type enzyme when AMP and substrate concen-

trations are below the optimum. It also seems to be of significance that glucose-6-phosphate, an allosteric inhibitor of phosphorylase *b*, inhibits the P_3 isoenzyme to a higher extent and the P_1 isoenzyme to a lesser extent only. The role of glucose-6-phosphate in feedback regulation is therefore different for the two isoenzymes (Vereb and Bot, 1972; Will and Krause, 1972).

Phosphorylation and dephosphorylation both play significant roles in the in vivo regulation of phosphorylase activity. As for the physiological role of isophosphorylases is concerned, the possibility that either the phosphorylation and/or dephosphorylation of the two isoenzymes occur at different rates or the two isoenzymes competitively inhibit each other's transformation cannot be ruled out yet.

Since antiphosphorylase inhibits the activity of not only phosphorylase *b* but also that of phosphorylase *a*, the immunotitration method seems to be suited for the investigation of these problems as well.

References

Davis, C. H., Olsgaard, R. B., Fischer, E. H., Krebs, E. G., (1964) Fed. Proc. *23* 2317
Davis, C. H., Schlieselfeld, L. H., Wolf, D. P., Leavitt, C. A., Krebs, E. G. (1967) J. Biol. Chem. *242* 4824—4833
Fischer, E. H., Krebs, E. G. (1958) J. Biol. Chem. *231* 65—71
Gornall, A. G., Bardawill, C. J., David, M. M. (1949) J. Biol. Chem. *177* 751—766
Hanabusa, K., Kohno, K. (1969) J. Biochem. (Tokyo) *66* 69—76
Henion, W. F., Sutherland, E. W. (1957) J. Biol. Chem. *224* 477—488
Illingworth, B., Cori, G. T. (1953) in Biochemical Preparations (Snell, E. E. ed), Vol. 3, pp. 1—9, John Wiley et Sons, Inc., New York
Jókay, I. (1963) Acta Microbiol. *10* 163—173
Jókay, I., Bot, Gy., Szilágyi, T. (1958) Acta Physiol. Acad. Sci. Hung. *14* 155—161
Jókay, I., Tóth, S. (1966) Z. Immun. forsch. *130* 17—29
Schlieselfeld, L. H., Davis, C. H., Krebs, E. G. (1970) Biochemistry, *9* 4959—4965
Schlieselfeld, L. H. (1973) Ann. New York Acad. Sci. *210* 181—191
Taussky, H. H., Shorr, E. (1953) J. Biol. Chem. *202* 675—685
Vereb, Gy., Csornai, M. (1970) in The 34th Annual Conference of the Hung. Physiol. Society. 1968 (ed. K. Lissák), Akadémiai Kiadó, Budapest, p. 190
Vereb, Gy., Bot, Gy., (1972) Acta Biochim. Biophys. Acad. Sci. Hung. *7* 35—46
Will, H., Krause, E. G., Wollenberger, A. (1970) Biochim. Biophys. Res. Comm. *40* 7—14
Will, H., Krause, E. G. (1972) Acta Biol. Med. Germ. *28* 919—933
Yunis, A. A., Fischer, E. H., Krebs, E. G. (1962) J. Biol. Chem. *237* 2809—2815

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Studies on Human Tonsillar Lymphocyte Membranes

I. Isolation and Characterization of a Membraneous Glycoprotein Fraction from Human Lymphocytes

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A microsomal fraction containing high amounts of plasma membrane has been isolated from human tonsillar lymphocytes. The subcellular fractions were characterized on the basis of their chemical composition and enzyme activities. A glycoprotein fraction solubilized by lithium-diiodo-salicylate (LIS) was purified by ion-exchange chromatography. The purified glycoprotein was analyzed by various methods and its carbohydrate content was determined. The glycoprotein contained large amounts of hexoses and sialic acid and this fraction represented 3—4% of the whole plasma membrane protein. The fraction was shown to consist of three individual proteins (4×10^4, 4.6×10^4 and 4.8×10^4 daltons) determined by SDS-polyacrylamide gel electrophoresis.

Introduction

The plasma membrane has several functions. This membrane is a barrier between the cytoplasm and the extracellular area, it plays an important role in transport processes, and many receptors and antigens are embedded in the surface membrane. In the case of the lymphocytes histocompatibility antigens (Medawar, 1956; Turner et al., 1972; Nathenson, Cullen, 1974), lectin receptors (Burger, 1973; Pospisilová et al., 1974) immunoglobulin receptors (Pierce et al., 1971; 1973) and antigen receptors (Unanue, 1971; Chiller et al., 1971; Unanue et al., 1973) are localized in the surface membrane. The plasma membrane of T-lymphocytes plays a role in spontaneous rosette formation (Brain et al., 1970, Coombs et al., 1970) and this phenomenon can be inhibited by various methods (Bach, Dardenne, 1971; Bach et al., 1969; Fekete et al., 1972; Kreeftenberg, Leerling, 1972; Bach, Dardenne, 1973; Wortis et al., 1973).

The plasma membrane has a fluid mosaic structure (Singer, Nicolson, 1972; Capaldi, 1974). Proteins are embedded in the phospholipid bilayer matrix. Some glycoprotein molecules span the membrane and their terminal parts occur on both surfaces of the lipid matrix (Bretscher, 1971; Marchesi et al., 1972).

The carbohydrate moieties of the membraneous glycoproteins and glyco-

Abbreviations: DEAE-, diethyl-amino-ethyl-; DNA, deoxyribonucleic acid; LIS, lithium diiodo-salicylate; NANA, N-acetyl-neuraminic acid; PHA, phytohemagglutinin; RNA, ribonucleic acid; RN-ase, ribonuclease; SDS, sodium dodecyl sulfate

lipids are very specific, determining the functions of the proteins or lipids (blood group antigens, virus receptors, lectin receptors etc.) The histocompatibility antigens (H-2 antigens in mouse and HL-A antigens in man) also contain carbohydrate units (McPherson et al., 1971; Schwartz et al., 1973; Nathenson, Cullen, 1974) but these carbonydrate units do not play any role in histocompatibility. Reisfeld et al. (1971, 1974) isolated these antigens as carbohydrate-free proteins.

The composition of the membrane proteins depends on the preparation method. The integral proteins from the membrane can be solubilized by special procedures (Hamaguchi, Cleve, 1972; Marchesi, Andrews, 1971; Marchesi et al., 1972) using lithium diiodo-salicylate extracted the "spanning" glycoprotein from human red blood cell membrane. This glycoprotein was shown to have MN antigen properties and to contain influenza virus and PHA-receptor sites (Marchesi, Andrews, 1971; Marchesi et al., 1972).

We have used this dissociating agent (LIS) for extracting glycoproteins from the plasma membrane and from the microsomal fraction of human tonsillar lymphocytes. The extracted glycoprotein fraction was further purified and the purified glycoprotein fraction was studied by analytical methods. The isolated glycoprotein fraction was found to inhibit the spontaneous rosette formation between human blood lymphocytes and sheep red blood cells (Hrabák et al., 1974) and to be a receptor-like protein for lectins (Hrabák et al., in preparation).

Materials and methods

All reagents were of analytical grade. Lithium diiodo-salicylate was prepared from salicylic acid and ICl, according to Woollet et al. (1943).

Cells and cell particles

The lymphocytes were isolated from freshly removed tonsils of 3–6-year old children by the method of Piffkó et al. (1970). The cell suspension was not separated further. The cells were centrifuged and disrupted by freezing and thawing three times (using a mixture of ethanol, dry ice and a water bath at 37 °C) and were homogenized in Hanks' medium diluted with water (1 : 1). The suspension was centrifuged at $600 \times g$ for 10 min to remove nuclei and undisrupted cells. The supernatant was centrifuged at $7000 \times g$ for 20 min to remove mitochondria, and the remaining supernatant was centrifuged at $45000 \times g$ for 60 min to remove the microsomal fraction according to Demus (1973). A flow sheet of the fractionation is shown in Fig. 1. The light microsomal fraction was isolated by centrifuging the postmicrosomal supernatant at $240{,}000 \times g$ for 60 min in the SW 41 rotor of a Beckman L2-65 B ultracentrifuge. The subcellular fractions were characterized on the basis of their marker enzymes and chemical composition. In some experiments the plasma membrane fraction was purified by Demus' method (1973), for comparing the data for purified plasma membrane with those for the microsomal fractions.

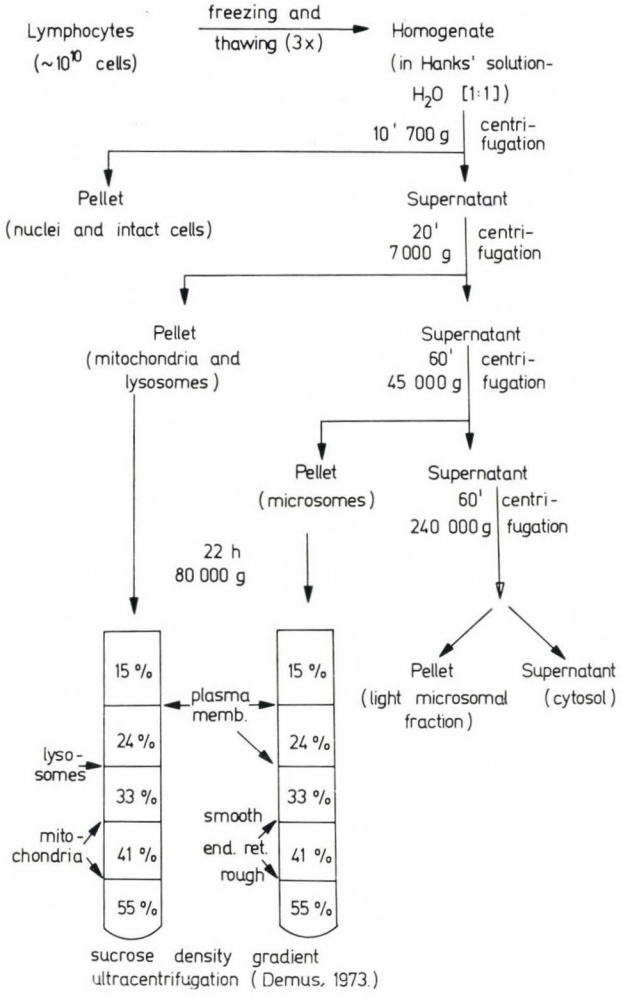

Fig. 1. Flow sheet of the fractionation of human tonsillar lymphocytes (Demus, 1973)

Enzyme assays

5'-nucleotidase activity was estimated according to Heppel and Hilmoe (1955), glucose-6-phosphatase activity was determined by the method of Swanson (1955), using acetate buffer instead of citrate. The β-glycerophosphatases were measured by the method of Gianetto and de Duve (1955), using acetate (pH 5.6) and Tris-HCl (pH 8.8) buffers. Cytochrome oxidase was estimated by the Nadi-reaction at pH 7.2 using 0.05 M phosphate buffer.

Chemical determinations

RNA and DNA were separated by a slightly modified method of Fleck and Munro (1962) DNA was determined according to Burton (1956) and RNA was measured by the method of Schneider (1957). We have used hydrolyzed DNA (from chicken blood) and D-ribose as standard. N-acetyl-neuraminic acid was measured according to Warren (1959) and Aminoff (1961) using NANA (N-acetyl-neuraminic acid) as standard. Total hexosamine content was determined according to Boas (1953) and Dische and Borenfreund (1950). Total neutral hexose content was measured using anthrone reagent (Roe, 1955), as standard glucose was employed. Protein was determined by Lowry's method (Lowry et al., 1951) with bovine serum albumin as a standard. Inorganic phosphate was measured according to Chen et al. (1956) with NaH_2PO_4 as a standard. Cholesterol was determined by the method of Stadtman (1957) using cholesterol as standard, the phospholipid content was measured on the basis of the inorganic phosphate content of the hydrolysate in perchloric acid (Marinetti et al., 1959).

Preparation of the glycoprotein

We prepared aceton-dry powder from microsomes and mitochondria and the powder was the source of the glycoprotein. The plasma membrane fraction was purified from microsomes by Demus' method (1973). 250 mg aceton-dry powder and plasma membrane were used for the LIS-treatment. The preparation was carried out according to Marchesi et al. (1961, 1972) and is shown in Fig. 2. The cation-exchange chromatography was carried out on cellulose-phosphate column (Na^+-form) using 20 mM NaH_2PO_4 for elution. The major part of the protein was eluted with the first peak, the residual protein could be eluted by 0.5 M NaH_2PO_4. The first fraction was further investigated.

Gel-filtration

A Sephadex G-150 column (1 × 95 cm) was used for gel filtration. The glycoprotein fraction (150–200 μg) was dissolved and eluted with 0.1 M ammonium formate and with 0.1 M ammonium formate+0.1 M 2-mercaptoethanol. Bovine serum albumin (M.wt. 6.8×10^4) and pancreatic RN-ase (M.wt. 1.4×10^4) were used for calibration. Protein concentration was estimated by measuring the absorbance of the solution at 280 nm.

DEAE-cellulose chromatography

150 μg glycoprotein was applied onto a DEAE-cellulose (Cl^--form) column (1.5 × 20 cm) and was eluted stepwise as follows: 10 mM, 100 mM, 500 mM tris–HCl (pH 7.5), 500 mM tris–HCl–1 M NaCl (pH 7.5) finally 1 M NaCl-1 M HCl. Absorbances at 280 nm.

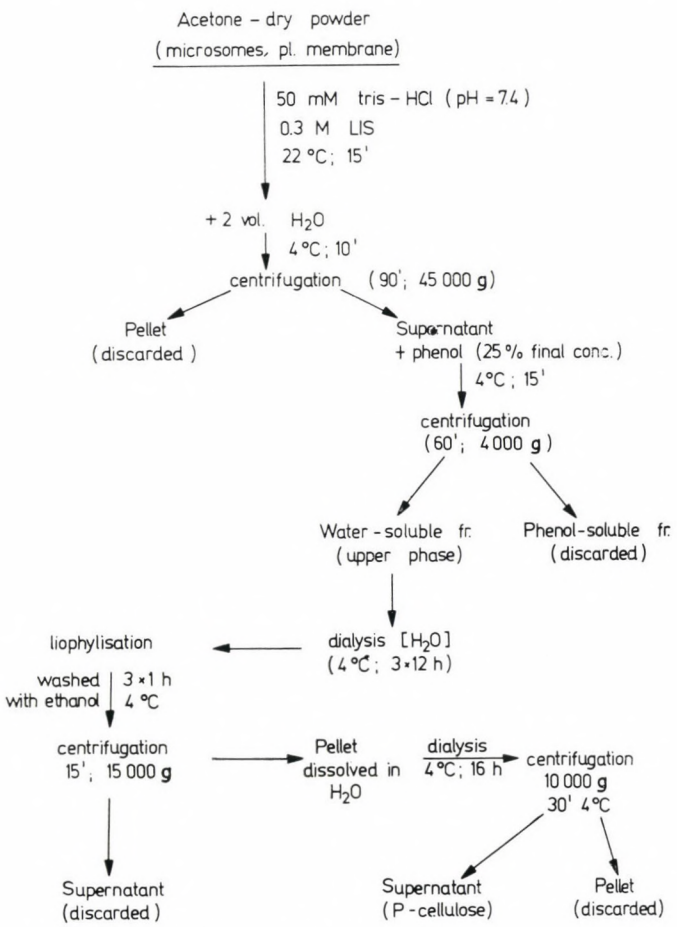

Fig. 2. Preparation of the membraneous glycoprotein fraction from subcellular fractions of human tonsillar lymphocytes

SDS-polyacrylamide gel electrophoresis

Electrophoresis was carried out in 7% gels containing 0.1% SDS (Weber, Osborn, 1969) at pH 8.3, in 0.025 M Tris – 0.192 M glycine at 5mA/gel for 100–120 minutes. Relative mobilities were expressed in terms of the mobility of the bromophenol blue marker and for the calculation of the molecular weights of the proteins RN-ase polymers prepared by diethylpyrocarbonate (Wolf et al., 1970) were used as standard.

After the electrophoretic run the gels were fixed in 50% TCA for 16 hours and were then stained by Coomassie brillant blue (0.1% solution in 50% TCA). The gels were destained by 7% acetic acid.

For electrophoresis the samples were heated at 100 °C for 5 minutes under reductive conditions (62 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol.)

Ion exchange thin layer chromatography

The qualitative estimation of hexosamines and amino acids was carried out by the simple method of Hrabák (1973) using Fixion 50×8 chromatoplates (Chinoin – Nagytétény).

Results

Cellular subfractions

Cell disruption and separation by differential centrifugation resulted in five subcellular fractions. These fractions were characterized on the basis of their enzymatic markers and chemical composition. Purified plasma membrane, endoplasmic reticulum and other subfractions can be prepared by discontinuous sucrose gradient ultracentrifugation of the mitochondrial and microsomal fractions (Demus, 1973). The procedure is shown in Fig. 1. The chemical composition and the activities of the marker enzymes of the subcellular fractions are summarized in Tables 1 and 2. The distribution of the protein content in the cellular subfractions is shown in Table 3. The purified plasma membrane represented only 1% of the cellular protein content (Demus, 1973). The nuclear fraction represented almost 50% of the whole cell protein. This result is inconsistent with those obtained by other preparative methods (Allan, Crumpton, 1970; Ferber et al., 1972; Demus 1973). Similarly our results concerning the protein content

Table 1

Chemical composition of the subcellular fractions of human tonsillar lymphocytes

Fraction	DNA	RNA	Cholesterol	P-lipid	Cholesterol P-lipid moles/moles	Hexose	Hexosamine* μg/mg protein	Sialic acid*
Homogenate	588	62.5	23.6	127	0.396	125(16.3)	14.6	6.72
Nuclei	*788*	67.0	16.9	133	0.256	103(49.2)	10.2	7.2
Mitochondria	51.5	96.8	82.1	280	0.588	89(32.4)	17.5	9.5
Microsomes	21.9	*132.0*	*136.6*	*365*	*0.754*	71(20.0)	*35.8*	*17.8*
Light microsomal fr.	42.6	131.1				17(–)		9.4
Cytosol	8.75	9.45	3.41	31.5	0.216	194(0.9)	5.0	4.92

* Determinations were carried out using aceton-dry powder, except for the cytosol fraction.
Data in parentheses refer to pellets after TCA-treatment.

Table 2

Specific activities of marker enzymes in lymphocyte subfractions

Fraction	5'-nucleotidase μmole P/mg protein/hour	Glucose-6-phosphatase μmole P/mg protein/hour	Phosphatase acid (pH=5.6) μmole P/mg protein/hour	Phosphatase alkaline (pH=8.8) μmole P/mg protein/hour	Cytochrome oxidase absorbance (500 nm)/mg protein/hour
Homogenate	1.46	1.11	1.01	0.11	4.9
Nuclei	1.05	1.15	0.85	0.44	3.1
Mitochondria	3.25	2.18	1.86	1.98	11.6
Microsomes	3.16	1.96	1.03	2.43	3.8
Light microsomal fraction	0.44	0.36	—	0.25	1.15
Cytosol	0	0.36	1.17	0	4.2

The details of determinations are given in the text. Cytochrome oxidase was measured on the basis of the Nadi-reaction at pH 7.2 in a phosphate buffer. The reaction was stopped by centrifuging the suspension. The concentration of p-phenylene-diimine was measured at 500 nm.

Table 3

The distribution of protein in the subcellular fractions of human tonsillar lymphocytes

	mg protein	Distribution in per cent of recovered protein
Homogenate	71.20	
Recovered protein	59.34	100
Nuclei	28.70	48±5
Mitochondria	7.44	10±2
Microsomes	1.80	3.5±1
Cytosol (including light microsomes)	21.40	35±4
Plasma membrane	0.40	0.8±0.15

of cytosol have shown lower values than those reported by the authors mentioned above. The mitochondrial and microsomal fractions represented 8–12 and 3–5%, respectively, of the whole cell protein, in agreement with the results of other authors.

Preparation of the glycoprotein fraction

The procedure is shown in Fig. 2. The results of the cellulose phosphate chromatography can be seen in Fig. 3. The first peak containing glycophorin-like glycoprotein was investigated further. 1 mg glycoprotein was purified from 200 mg aceton-dry powder of the microsomal fraction. According to the principles

of cellulose phosphate chromatography, the purified fraction consisted of negatively charged component(s).

The glycoprotein fraction represented about 3.5% of the plasma membrane protein. As indicated in Table 4, this glycoprotein formed 0.0035% of the whole cell protein, and the plasma membrane represented only 1% of the cellular total protein content (Demus, 1973).

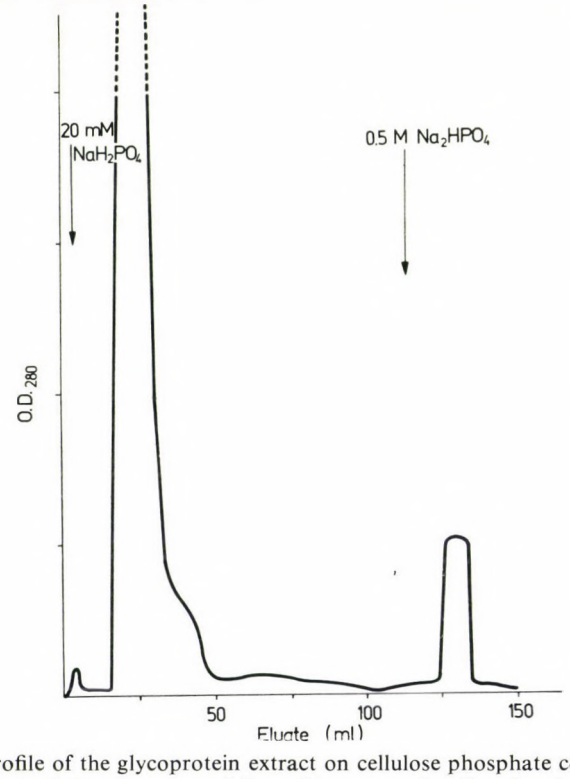

Fig. 3. Elution profile of the glycoprotein extract on cellulose phosphate column (2×25 cm). The protein content of the eluate was followed by measuring the absorbance at 280 nm of the 5 ml fractions

DEAE-cellulose chromatography and gel filtration experiments

The results of DEAE-cellulose chromatography supported those obtained by cellulose phosphate chromatography. The glycoprotein fraction was eluted in two peaks of strong negative charge (Fig. 4).

The samples gave identical patterns upon elution with 0.1 M ammonium formate and 0.1 M ammonium formate +0.1 M mercaptoethanol from a Sephadex G-150 column. The samples were eluted in a single peak containing material of a 5×10^4 daltons molecular weight (Fig. 5). The identical patterns support the view that the glycoprotein fraction does not contain polypeptide chains held together by disulfide bonds.

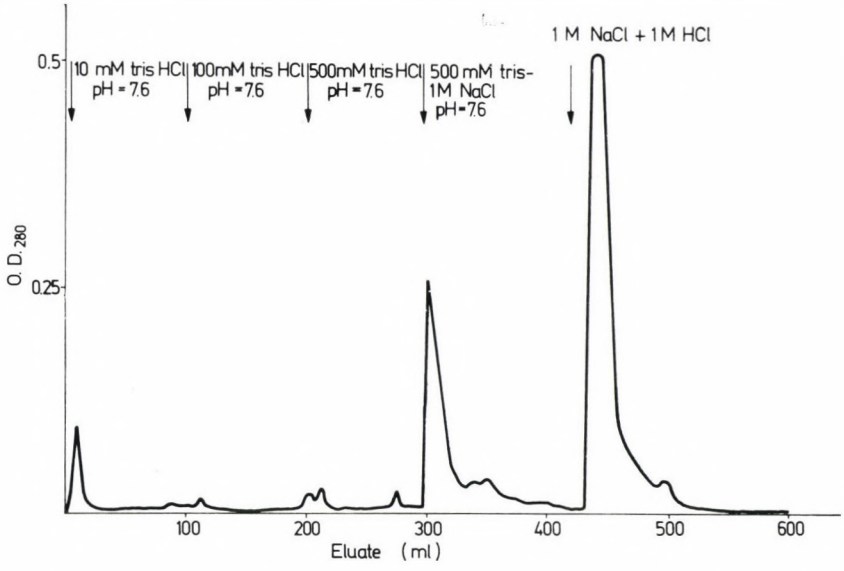

Fig. 4. Stepwise elution profile of the glycoprotein fraction on DEAE-cellulose column (1.5 × 20 cm). The protein content of the eluate was followed by measuring the absorbance at 280 nm of the 5 ml fractions

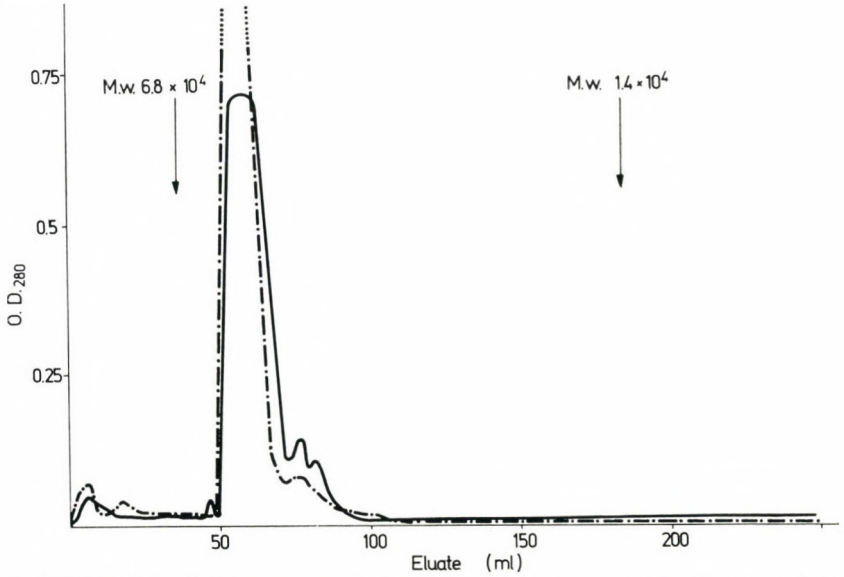

Fig. 5. Gel filtration of the glycoprotein fraction on Sephadex G-150 column (1 × 95 cm). 150 – 200 μg glycoprotein was applied onto the column and was eluted with 0.1 M ammonium formate (continuous line) and 0.1 M ammonium formate-0.1 M mercapto-ethanol (dotted line). The arrows indicate the location of the calibrating proteins (bovine serum albumin M. wt. 69 000 and ribonuclease M. wt. 14 000)

SDS-polyacrylamide gel electrophoresis

Gel-electrophoresis was carried out according to Weber and Osborn (1969) to test the homogeneity of the glycoprotein fraction and to estimate the molecular weights of its components. The LIS-extracted glycoproteins of the plasma membrane, of the microsomal and the mitochondrial fractions were investigated. The microsomal and plasma membrane glycoproteins gave identical patterns. Two bands were distinctly visible in both cases, however, the band containing material of lower molecular weight was a double band whose presence may be attributed to the microheterogeneity of glycoproteins, or else, it could represent two identical proteins. The molecular weights were: 4×10^4, 4.6×10^4 and 4.8×10^4 daltons (the gel filtration experiments had shown 5×10^4 daltons). Calibration curves were prepared as described in Materials and Methods. The mitochondrial LIS-extract contained some additional protein bands besides the microsomal band (Fig. 6).

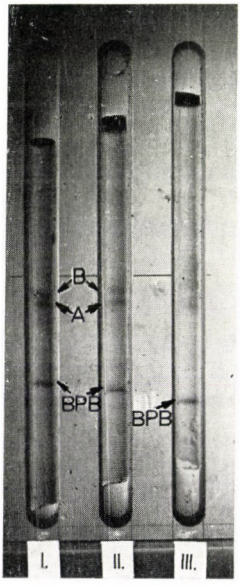

Fig. 6. SDS-polyacrylamide gel electrophoretic patterns of the glycoprotein fractions from microsomal, mitochondrial and plasma membrane fractions of human tonsillar lymphocytes. The details are given in the text. I, plasma membrane LIS-glycoprotein; II, microsomal LIS-glycoprotein; III, mitochondrial LIS-glycoprotein. BPB, bromophenol blue marker; A, plasma membrane glycoprotein M. wt. 4×10^4; B, double band consisting of two individual polypeptides M. wt. 4.6×10^4 and $4.8 \times 10.^4$) The gels were stained with 0.1% Coomassie brillant blue in 50% TCA and destained in 7% acetic acid. The calculation of molecular weights was based on the relative mobilities of the bands compared to those of RN-ase polymers (Wolf et al., 1970). The relative mobilities were referred to the bromophenol blue marker

Carbohydrate composition

The carbohydrate composition of the microsomal LIS-extract is shown in Table 4. The extract contained high amounts of neutral hexoses and sialic acid. This high sialic acid content may, at least partly, be responsible for the strong negative charge (the carboxyl group of sialic acid is a stronger acidic than that of amino acids). Hexosamine was mainly present in the form of glucosamine derivatives. The presence of galactosamine derivatives is supported by the PHA-receptor activity of the glycoprotein fraction (Hrabák et al., in preparation) because N-acetyl-D-galactosamine is a receptor of PHA (phytohaemagglutinin) (Sharon, Lis, 1972). The qualitative amino acid composition was estimated also by ion-exchange thin-layer chromatography: basic and acidic amino acids, leucine, threonine, serine and glycine were present in high amounts in the hydrolysate of the glycoprotein fraction.

Table 4

The carbohydrate composition and protein content of LIS-solubilized glycoprotein fraction

Data in parentheses refer to pellets after TCA-treatment. The carbohydrate content of LIS-extract was measured in aqueous solutions

Fraction	Protein %	Hexose μg/mg protein	Hexosamine μg/mg protein	Sialic acid
Homogenate	100	125(16.3)	14.6	6.72
Microsomes	3.5 ± 1	71(20.0)	35.8	17.8
LIS-extract	N.D.	520	N.D.	95.5
LIS-glycoprotein purified on P-cellulose	0.035	278	39.5	122.0

Discussion

For the isolation of lymphocyta plasma membranes (Allan, Crumpton, 1970, 1972; Demus, 1973; Ferber et al., 1972; van Blitterswijk et al., 1973; Smith et al., 1975) different methods have been worked out. These methods consist of characteristic steps: cell isolation from tissues, cell disruption, differential centrifugation and gradient ultracentrifugation. The characterization of cellular subfractions is based on a study of chemical and enzymatic markers and on ultrastructural investigation.

Our microsomal and mitochondrial fractions had a high 5'-nucleotidase activities. This enzyme is a market of the plasma membrane. The lighter subfractions of microsomal and mitochondrial fractions were also rich in this enzyme (Demus, 1973). Therefore these fractions were used for plasma membrane investigations. In most cases microsomal aceton-dry powder was chosen as a source of glycoprotein in order to losses during ultracentrifugation. The glycoprotein

fractions of microsomes and plasma membrane gave identical SDS-polyacrylamide electrophoretic patterns.

The microsomal fraction contained endoplasmic reticulum (glucose-6-phosphatase activity was high) beside the plasma membrane. Alkaline phosphatase is a marker of plasma membrane in some types of cells (Wallach, Winzler, 1974) and in lymphocytes we found it mainly in the plasma membrane fraction (unpublished results). The mitochondrial fraction contained mitochondria (high specific activity of cytochrome oxidase), lysosomes (acidic β-glycerophosphatase), endoplasmic reticulum (high activity of glucose-6-phosphatase and high content of RNA) and plasma membrane (5'-nucleotidase activity). Our results are similar to those of Demus (1973). This author succeeded in isolating plasma membrane from both fractions, endoplasmic reticulum from microsomes and lysosomes and purified mitochondria from mitochondrial fraction.

In our preparations the carbohydrates, mainly sialic acid, were present in highest amount in the microsomal fraction. Since sialic acid is known to be a characteristic component of plasma membranes, this observation also supports the fact that the microsomal fraction contains a high amount of plasma membranes. This fraction contained the highest cholesterol to phospholipid ratio, characteristic of the plasma membrane (Demus, 1973).

Our results concerning the protein content of nuclear and cytosol fractions may be attributed to the clumping of the nuclear fraction brought about by a partial release of DNA from nuclei during cell disruption. The clumped nuclear fraction may adsorb soluble cytoplasmic proteins and this may lead to a virtual increase in the protein content of the nuclear fraction accompanied by a concomitant decrease of the protein content of the cytosol. The mitochondrial and microsomal proteins were not influenced by this effect, and the data agree with those of others.

Lithium-diiodo-salicylate is a chaotropic anionic detergent. LIS solubilizes the membraneous proteins and facilitates further purification (Rosai et al., 1971; Marchesi, Andrews, 1971). LIS was used for the isolation from different types of cells of glycoproteins rich in carbohydrates (Marchesi, Andrews, 1971; Hourani et al., 1973; Hrabák et al., 1974). The human erythrocyte glycophorin (Marchesi, Andrews, 1971; Marchesi et al., 1972; Segrest et al., 1972) was found to be responsible for the MN antigenic site and for receptor sites of influenza virus and lectins on the surface of red blood cells. Hourani et al. (1973) solubilized a glycoprotein fraction (consisting of our glycoproteins) from mouse L 1210 leukemic cells. This glycoprotein served as a receptor for different lectins (wheat germ agglutinin, Concanavalin A, Lens culinaris lectin).

The lymphocytic glycoprotein was similar to glycophorin in physico-chemical properties: strong negative charge, molecular weight approximately 5×10^4, high sialic acid content. Glycophorin is however homogeneous (recently Furthmayr et al. (1975) have reported subfractions of glycophorin with molecular weights lower than 5×10^4. Glycophorin contains 60% carbohydrate, our glycoprotein contains 278 μg hexose and 122 μg sialic acid per mg protein. The L 1210

cell glycoprotein also contains high amounts of various sugars (Hourani et al., 1973).

Our microsomal and membraneous glycoprotein fractions consist of more than one polypeptide. SDS-polyacrylamide electrophoretic studies have revealed the presence of three polypeptides of a molecular weight of 4×10^4, 4.6×10^4 and 4.8×10^4 respectively.

Attempts were made to find out the biological role and properties of the lymphocytic glycoprotein fraction. Previously we have reported that spontaneous rosette formation is inhibited by our glycoprotein (Hrabák et al., 1974). Now we intend to study the lectin-receptor property of this glycoprotein. Details of these experiments will be published elsewhere.

References

Allan, D., Crumpton, J. (1970) Biochem. J. *120* 133
Allan, D. and Crumpton, J. (1972) Biochim. Biophys. Acta *274* 22
Aminoff, D. (1961) Biochem. J. *81* 384
Bach, J. F., Dardenne, M. (1971) Rev. Europ. Études Clin. Biol. *16* 770
Bach, J. F., Dormont, J., Dardenne, M., Balner, H. (1969) Transplantation *8* 265
Bach, J. F., Dardenne, M. (1973) Cell. Immunol. *6* 394
van Blitterswijk, W. J., Emmelot, P., Feltkamp, C. A. (1973) Biochim. Biophys. Acta *298* 577
Boas, N. F. (1953) J. Biol. Chem. *204* 553
Brain, P., Gordon, J., Willett, S. (1970) Clin. Esp. Immunol. *6* 681
Bretscher, M. S. (1971) J. Mol. Biol. *59* 351
Burger, M. M. (1973) Fed. Proc. *32* 91
Burton, K. (1956) Biochem. J. *62* 315
Capaldi, R. A. (1974) Sci. Amer. *230* 26
Chen, P. S., Toribara, T. Y., Warner, H. (1956) Anal. Chem. *28* 1756
Chiller, J. M., Habicht, G. S., Weigle, W. O. (1971) Science, *171* 813
Coombs, R. R. A., Gurner, B. W., Wilson, A. B., Holm, G., Lindgren B. (1970) Int. Arc. Allergy *39* 658
Demus, H. (1973) Biochim. Biophys. Acta *291* 93
Dische, Z., Borenfreind, E. (1950) J. Biol. Chem. *184* 517
Fekete, B., Szegedi, Gy., Gergely, P., Szabó, G., Petrányi, Gy. (1972) Acta Med. Acad. Sci. Hung. *29* 327
Ferber, E., Resch, K., Wallach, D. F. H., Imm, W. (1972) Biochim. Biophys. Acta *266* 494
Fleck, A., and Munro, H. N. (1962) Biochim. Biophys. Acta. *55* 571
Furthayr, H., Tonita, M., Marchesi, V. T. (1975) Biochem. Biophys. Res. Commun. *65* 113
Gianetto, R. and de Duve, C. (1955) Biochem. J. *59* 433
Hamaguchi, H., Cleve, H. (1972) Biochim. Biophys. Acta *278* 271
Heppel, L., Hilmoe, R. J. (1955) Methods in Enzymol. *2* 547
Hourani, B. T., Chace, N. M., Pincus, J. H. (1973) Biochim. Biophys. Acta *328* 520
Hrabák, A. (1973) J. Chromatog. *84* 204
Hrabák, A., Antoni, F., T. Szabó, M., Merétey, K. (1974) 9th FEBS Meeting Budapest, 1974, Abstract S6a20
Kreeftenberg, J. G., Leerling, M. F. (1972) *14* 665
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. *193* 265
Marchesi, V. T., Andrews, E. P. (1971) Science *174* 1247

Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P. Scott, R. E. (1972) Proc. Natl. Acad. Sci. USA *69* 1445
Marinetti, G. V., Erbland, J., Stotz, E. (1959) Biochim. Biophys. Acta *31* 251
McPherson, J. C., Clamp, J. R., Manstone, A. J. (1971) Biochemistry *8* 225
Medawar, P. B. (1956—1957) Harvey Lectures 52 pp. 144—176
Nathenson, S. G., Cullen, S. E. (1974) Biochim. Biophys. Acta *344* 1
Pierce, C. W., Solliday, S. M., Asofsky, R. (1971) J. Exptl. Med. *134* 395
Pierce, C. W., Asofsky, R., Solliday, S. M. (1973) Fed. Proc. *32* 41
Piffkó, P., Köteles, G., Antoni, F. (1970) Pract. Otorhinolaryng. *32* 305
Pospisilová, J., Haskoveč, C., Entlicher, G., Kocourek, J. (1974) Biochim. Biophys. Acta *373* 444
Reisfeld, R. A., Pellegrino, M. A., Kahan, B. D. (1971) Science, *172* 1134
Reisfeld, R. A., Ferrone, S., Pellegrino, M. A. (1974) Isolation and serological evaluation of HL-A antigens solubilized from cultured human lymphoid cells. Methods in Membrane Biology *1* 143, ed. by Korn, E. D. Plenum New York, 1974
Roe, J. M. (1955) J. Biol. Chem. *212* 335
Rosai, J., Tillack, T. W., Marchesi, V. T. (1971) Fed. Proc. *30* 453
Schwartz, B. D., Kato, K., Cullen, S. E., Nathenson, S. G. (1973) Biochemistry *12* 2157
Schneider, W. C. (1957) Methods in Enzymol. *3* 680
Segrest, J. P., Jackson, R. L., Marchesi, V. T., Guyer, R. B., Terry, W. (1972) Biochem. Biophys. Res. Commun. *49* 964
Sharon, N., Lis, H. (1972) Science *177* 949
Singer, S. J., Nicolson, G. L. (1972) Science *175* 720
Smith, W. I., Ladoulis, C. T., Misra, D. N., Gill, T. J., Baizin, K. (1975) Biochim. Biophys. Acta *382* 506
Stadtman, T. C. (1957) Methods in Enzymol. *3* 392
Swanson, M. A. (1955) Methods in Enzymol. *2* 541
Turner, M. J., Strominger, J. L., Sanderson, A. R. (1972) Proc. Natl. Acad. Sci. USA *69* 200
Unanue, E. R. (1971) J. Immunol. *107* 1168
Unanue, E. R., Engers, H. D., Karnovsky, M. J. (1973) Fed. Proc. *32* 44
Wallach, D. F. H., Winzler, R. J. (1974) Evolving Strategies and Tactics in Membrane Research, Springer Verlag, N. Y. 1974
Warren, L. (1959) J. Biol. Chem. *234* 1971
Weber, K., Osborn, M. (1969) J. Biol. Chem. *244* 4406
Wolf, B., Lausarot, P., Lesnaw, J. A., Reichmann, M. E. (1970) Biochim. Biophys. Acta *200* 180
Woollet, J., Johnson, A. (1943) Org. Syntheses Coll. Volume II. 343
Wortis, H. H., Cooper, A. G., Brown, M. C. (1973) Nature N. B. *243* 109

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Vinblastine Inhibition of Microtubule Assembly *In vitro*

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The effect of vinblastine on the *in vitro* polymerization of tubulin was investigated. It has been shown that the presence of VLB prevents the assembly of tubulin in rat brain extracts, and the addition of VLB to assembled microtubules results in their rapid depolymerization. The polymerization was monitored by viscometry and electron microscopy.

Introduction

Weisenberg (1972) has described the conditions necessary for the *in vitro* repolymerization of tubulin into microtubules in partially purified rat brain extracts. In such an *in vitro* system, the factors which affect the polymerization and depolymerization of neuronal microtubules can be investigated with greater precision than it was previously possible in *in vivo* systems. The extent of polymerization can be followed quantitatively by measuring the changes in the turbidity (Borisy, Olmsted, 1972; Shelanski et al., 1973; Lee et al., 1974), flow birefringence (Haga et al., 1974), intrinsic tryptophan fluorescence (Staprans et al. 1975) or viscosity (Olmsted, Borisy, 1973; Kuriyama, Sakai, 1974; Haschke et al., 1974) of the tubulin solution.

Vinblastine, a potent mitotic inhibitory alkaloid of the periwinkle (*Catharanthus roseus* G. Don), is known to bind to tubulin (Bryan, 1972; Owellen et al., 1972; 1974) and to disrupt cytoplasmic microtubules *in vivo* (Malawista et al., 1968).

The aim of our experiments was to investigate the direct effect of vinblastine on *in vitro* repolymerized microtubules and on the polymerization of tubulin.

Materials and methods

Brain extract

Young male Wistar rats weighing about 160 g were killed by cervical dislocation. The whole brains were removed immediately. All subsequent operations were carried out at 0—4 °C. The thoroughly washed brains were minced with scissors

Abbreviations used: PIPES, 1,4-piperazine-bis-(ethanesulfonic acid); EGTA, ethyleneglycol-bis) (β-amino-ethyl ether) N,N'-tetraacetic acid; GTP, guanosine 5'-triphosphate; VLB, vinblastine sulfate.

and then homogenized in 1.5 ml reassembly buffer (100 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.6) per gram of tissue with a motor driven glass-Teflon homogenizer. The homogenate was centrifuged at 100,000 *g* for 30 minutes. The supernatant was used for the experiments.

Viscometry

Viscosity was measured in capillary viscometers immersed in a water bath regulated at 37 ± 0.1 °C. The flow times for the buffer ranged from 48 to 54 sec. The data are expressed as specific viscosities (η_{sp}).

Electron microscopy

Electron microscopic observations assured the specificity of polymerization. The samples were diluted tenfold in a microtubule stabilizing medium (20 mM phosphate buffer, 1 M hexylene glycol, pH 6.4) (Kirkpatrick et al., 1970). A drop of this mixture was placed on a grid coated with Formvar and carbon, negatively stained with 1% uranyl acetate, and examined with a model JEM 6C electron microscope (JEOL).

The protein concentration of the extracts was determined by the method of Lowry et al., as modified by Hartree (1972), using bovine serum albumin as a standard. The protein content of the extracts varied in the range of 12.0 – 14.5 mg/ml.

Results and discussion

After warming the sample to 37 °C, the viscosity gradually increased. A plateau was reached after 10 – 12 minutes and this level slowly decreased with continued incubation (Fig. 1).

When VLB was added to the extract prior to incubation at 37°C, the viscosity remained at the starting level (Fig. 1). 1 μM VLB was sufficient to fully prevent repolymerization. When VLB (10 μM) was added to the assembled microtubules the treatment resulted in an immediate and marked decrease of viscosity (Fig. 1). Some intact microtubules could be observed by electron microscopy, even after 20 minutes of exposure to VLB. They were, however, strongly reduced in number and length.

The effect of colchicine on the *in vitro* repolymerized microtubules is controversial. Olmsted and Borisy (1973) and Haga et al. (1974) observed only partial and slow degradation of microtubules after colchicine treatment (100 μM). In the experiments of Kuriyama and Sakai (1974) the addition of 100 μM colchicine caused a rapid and very marked decrease of the viscosity. Lee et al. (1974) could not observe any effect of colchicine (up to 100 μM) on the *in vitro* assembled microtubules. Preincubation with colchicine in all of these experiments prevented tubulin polymerization. Under our experimental conditions, the effect of colchicine

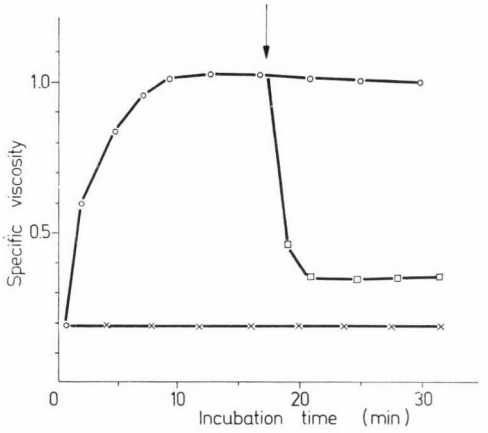

Fig. 1. The assembly of tubulin as followed by viscometry. Polymerization was carried out in the standard reassembly buffer (see text) at 37°C (○—○—○) VLB (1 μM) was added before incubation (×—×—×) or at the arrow 10 μM (—□—□—) to the reassembled mictotubules. The protein concentration was 12.7 mg/ml

(100 μM) was very similar to that of VLB and to the results obtained by Kuriyama and Sakai (1974).

Though VLB and colchicine have different binding sites on tubulin (Bryan, 1972; Krishan, Hsu, 1971), their mechanisms of action show marked similarities in these experiments.

We wish to thank Miss Zs. Suhajda for her expert technical help.

References

Borisy, G. G., Olmsted, J. B. (1972) Science *177* 1196—1197
Bryan, J. (1972) Biochemistry *11* 2611—2616
Haga, T., Abe, T., Kurokawa, M. (1974) FEBS Letters *39* 291—295
Hartree, E. F. (1972) Anal. Biochem. *48* 422—427
Haschke, R. H., Byers, M. R., Fink, M. R. (1974) J. Neurochem. *22* 837—843
Kirkpatrick, J. B., Hyams, L., Thomas, V. L., Howley, P. M. (1970) J. Cell. Biol. *47* 384—391
Krishan, A., Hsu, D. (1971) J. Cell. Biol. *48* 407—410
Kuriyama, R., Sakai, H. (1974) J. Biochem. *75* 463—471
Lee, Y. C., Samson, F. E., Houston, L. L., Himes, R. H. (1974) J. Neurobiol. *5* 317—330
Malawista, S. E., Bensch, K. G., Sato, H. (1968) Science *160* 770—771
Olmsted, J. B., Borisy, G. G. (1973) Biochemistry *12* 4282—4289
Owellen, R. J., Donigian, D. W., Hartke, C. A., Dickerson, R. M., Kuhar, M. J. (1974) Cancer Res. *34* 3180—3186
Owellen, R. J., Owens, A. H., Donigian, D. W. (1972) Biochem. Biophys. Res. Commun. *47* 683—691
Shelanski, M. L., Gaskin, F., Cantor, C. R. (1973) Proc. Natl. Acad. Sci. U. S. *70* 765—768
Staprans, I., Kenney, W. C., Dirksen, E. R. (1975) Biochem. Biophys. Res. Commun. *62* 92—97
Weisenberg, R. C. (1972) Science *177* 1104—1105

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Bound Water in Biology*

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A detailed investigation of the spin-diffusion coefficient D_s of water protons in skeletal muscle has been studied by pulsed nuclear magnetic resonance (NMR) methods. Skeletal muscles of mature male rats were placed in a sample holder in which the diffusion coefficient (D_s) of water could be determined as a function of fiber axis Θ. The value of $D_s(\Theta)$ was determined for $\Theta = 0°$, 45°, and 90°. The measured anisotropy $D_s(0)/D_s(90)$ was 1.39, and the value of $D_s(0)$ was 1.39×10^{-5} cm^2/sec. These results are interpreted within the framework of a model calculation in which the diffusion equation is solved for a regular hexagonal network similar to the actin-myosin filament network. The large anisotropy, and the large reduction in the value of D_s measured parallel to the filament axes lead to three major conclusions: (1) interpretations in which the reduction in D_s is ascribed to the effect of geometrical obstructions on the diffusion of "free" water are ruled out; (2) there is a large fraction of the cellular water bound or otherwise associated with the proteins in such a way that its diffusion coefficient is substantially reduced; and (3) cellular water cannot be considered to be equivalent to a dilute solution.

Introduction

Biological tissues are comprised of 60–90 per cent water by weight and the importance of water in the living system is undeniable. Although water has always been a subject of concern and curiosity to the biologist, very little basic knowledge is available on cellular water. An adequate model of the living cell, describing the unequal distribution of ions between the cellular inside and outside, the dynamic aspects of solute and water movement into and out of cells, and the bioelectric phenomena of cells requires that fundamental assumptions be made relative to the physical state of cellular water. The ultimate understanding of cellular function will indeed require concrete knowledge of the physical state of water.

Traditionally, biologists suggest that a living cell is equivalent to a dilute aqueous solution surrounded by a semipermeable membrane (e.g. Katz, 1966). Bioelectric phenomena of cells are also described classically by equations evolving

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essentially from dilute solution theory. The history of osmotic pressure, as the underlying mechanism for the movement of water in biological cells, dates from the 18th century. The greatest impetus to the application of osmotic theory to biological cells came, however, near the turn of the 20th century when chemists like van't Hoff and Arrhenius laid much of the foundation for dilute solution theory. Bernstein's theory of bioelectric phenomena evolves from the use of ideal solution theory; for example, Katz (p. 57) states that "in 1902 he (Bernstein) proposed an important theory which applied the physicochemical concepts of Nernst and Ostwald to bioelectric phenomena". Hodgkin and Huxley (1939) challenged the Bernstein theory which argued for the impermeability of the cell to sodium. Later, Hodgkin and others modified and extended the Bernstein theory to what is essentially the present concept of the excitable cell, yet the theory of dilute solutions remains fundamental to the membrane concept (Hodgkin, 1951).

It is also known that theories have existed which maintain that water in cytoplasm is not free as in dilute solutions but bound to cell constituents. The concept of bound water and electrolytes in living tissues continued to be pursued actively during the 1920's and 1930's (Rubner, 1922; Thoenes, 1925; Ernst, 1934; Gortner, 1934). It is difficult to know exactly why the bound water concept lost favor, however, two publications appear to be linked to its decline. First, A. V. Hill (1930) published a report based on vapor-pressure measurements that little, if any, water in blood or muscle is bound. This paper seems to have been taken as definitive even though Ernst adequately challenged this work in 1934. Second, Blanchard (1940) wrote a review on this subject which argued that ethylene glycol diffuses throughout the volume of erythrocyte cell water. He further maintained that since water could be supercooled to $-20\,^{\circ}C$, incomplete freezing of water might be attributed to supercooling rather than to bound water as Gortner and his colleagues had proposed. According to Ling (1969), "much of Blanchard's evidence against the bound water concept was eventually proven to be equivocal. Hydration of proteins became established by a large variety of modern techniques, described later. Nevertheless, Blanchard's review was a landmark in the history of cell physiology which thereafter saw the virtual abandonment of the hydration theory of cell water".

Since the 1950's, a small but persistent group of biological scientists have argued that a large part of, or all the cellular water exists in a physical state significantly different from ordinary water (Ernst et al., 1950; Troshin, 1966; Ling, 1962). Thus, two schools of thought have emerged concerning the physical state of cellular water, and hence, two schools of thought concerning transport and accumulation of solutes in living cells. A review of this fundamental controversy was presented by Oscar Hechter (1965) whereupon he concluded the following: "We have discussed the two opposing classical concepts of transport. Upon analysis, both ideas are shown to be right in part; and both partially wrong. The proponents of the plasma membrane thesis of transport were wrong in that they neglected the role of the cell interior, the holists were wrong in their de-emphasis of the plasma membrane and of intracellular membranes generally. If one con-

siders that membrane systems throughout the cell are involved in transport, and that cell exhibit diversity as well as uniformity, a pluralistic resolution is achieved."

To date, considerable evidence has been gathered by proponents of both views but little has been done to bring about the pluralistic resolution so proposed by Hechter.

A fundamental understanding of the physical state of cellular water will lead to a resolution of these divergent views (e.g. Ernst, 1970). It is, therefore, essential that we address ourselves to this task rather than considering the subject either unimportant or already solved.

Recent Studies on the Physical State of Water in Cells Utilizing Nuclear Magnetic Resonance Spectroscopy

(these papers are marked with asteriks)

Perhaps the first published experiments on living tissue, using NMR techniques, was that of Odeblad and his co-workers in 1956. These workers along with a host of other's have found that the relaxation times of tissue water protons are significantly reduced when compared to that of pure water or dilute electrolyte solutions. In addition, the spin diffusion coefficient of tissue water has been found to be reduced when comparison to pure water is made. These parameters (T_1, the spin lattice relaxation time; T_2, the spin-spin relaxation time; and D_s, the self diffusion coefficient) have been measured on a variety of biological tissues including nerve, muscle, brain, liver, spleen, kidney, lens, tendon, lung and heart, as well as in various benign and malignant tumors. Allowing for the wide range of sample types, the studies have all produced values of T_2 reduced by a factor of 30 to 60, T_1 reduced by a factor of 4 to 10, and D_s reduced by a factor of about 2 when compared to the values of these parameters obtained for pure water or dilute electrolyte solutions.

These data have been interpreted by some investigators as being consistent with the concept of "ordering" of the intracellular water (i.e. shorter relaxation times imply longer correlation times and thus indicate a more ordered state). Other investigators have proposed alternative explanations of the change in these parameters. The dominate hypothesis among opponents to the idea of changes in the physical properties of intracellular water is that the reduction in the NMR relaxation times can be explained by a two-phase fast-exchange model (Zimmerman, 1957). In this phenomenological model, the vast majority of the water is assumed to behave as if it were an ideal solution and the short relaxation times are attributed to the averaging effects of fast exchange of a majority of free water with a small fraction of water molecules tightly bound to intracellular macromolecules. The observation by many investigators of relaxation data fit by a single exponential has been cited as evidence in support of this interpretation. It is also pointed out that the water-ice transition reduces D by about 4 orders of magni-

tude (Eisenberg, 1969), so that a reduction of 40–60 per cent in the diffusion coefficient does not indicate much "ordering".

Since the fast-exchange hypothesis assumes that the vast majority of the intracellular water is "free", the observed diffusion coefficient should be essentially that of pure water. The observed reduction in D_s is therefore attributed to compartmentalization by the membrane systems of the cell, or to geometrical obstruction effects due to interaction of the water with biopolymers or other diffusion barriers within the cell (Wang, 1954). This interpretation persists even though Chang et al. have presented experimental evidence that the compartmentalization effects cannot account for the reduction of the diffusion coefficient in skeletal muscle (Chang, Hazlewood, 1975). In addition, preliminary reports by Rorschach et al. and Cleveland et al. on the self-diffusion coefficient of water protons in skeletal muscle suggested that geometrical obstruction effects are also inadequate for this purpose (Rorschach et al., 1973; Cleveland et al., 1973).

In order to better determine which hypothesis of cellular water is more nearly correct, it becomes necessary to determine the significance of the reduction of the diffusion coefficient of water. For example, if D_3 of cell water is reduced because of obstructive barriers then the simple two phase model would gain support which, in turn, would lend support to the concept that very little water is bound within living cells. On the other hand, if the reduction of D_s of cellular water cannot be accounted for by simple obstructive barriers then we are forced to conclude a larger fraction of cellular water may either be bound or otherwise experiencing some sort of long range forces within the cell. The remainder of my paper will be concerned with such a detailed study of diffusion conducted in our laboratory.

Description of experiment

Diffusion coefficients were determined using standard techniques of pulsed NMR. Samples in this study were prepared from the tibialis anterior of mature male rats (Texas Inbred) weighing between 350 and 500 grams. A reliable method for insuring muscle fiber orientation was developed and the details of this procedure may be found elsewhere. The method involved placement of the sample tube in the NMR probe and the orientation of the fiber axis, determined within $\pm 3°$, was noted on the top of the sample tube. The orientation of the fiber axis, relative to the magnetic field and its gradient, was adjusted by rotating a 12 mm sample tube manually.

Results and discussion

The results of these experiments are shown in Table 1 and plotted in Figure 1. Results of measurements of the self diffusion coefficient for pure bulk water and for randomized tibialis anterior are also included in Table 1. The value of the spin diffusion coefficient D_s for randomized muscle was obtained from two succes-

Table 1

Measurements of the Diffusion Coefficient Versus Muscle Fiber Orientation

Diffusion coefficient for pure bulk water 2.28×10^{-5}cm^2/sec
Diffusion coefficient for randomized muscle 1.19×10^{-5}cm^2/sec

	Diffusion Coefficients ($\times 10^{-5}$cm^2/sec)			
Sample	$=0°$	$=45°$	$=90°$	$D_s(90)/D_s(0)$
1	1.37	1.18	1.02	0.75
2	1.39	1.18	1.03	0.74
3	1.40	1.23	1.03	0.74
4	1.41	1.22	0.99	0.70
5	1.40	1.19	0.97	0.69
Average	1.39	1.20	1.01	0.72

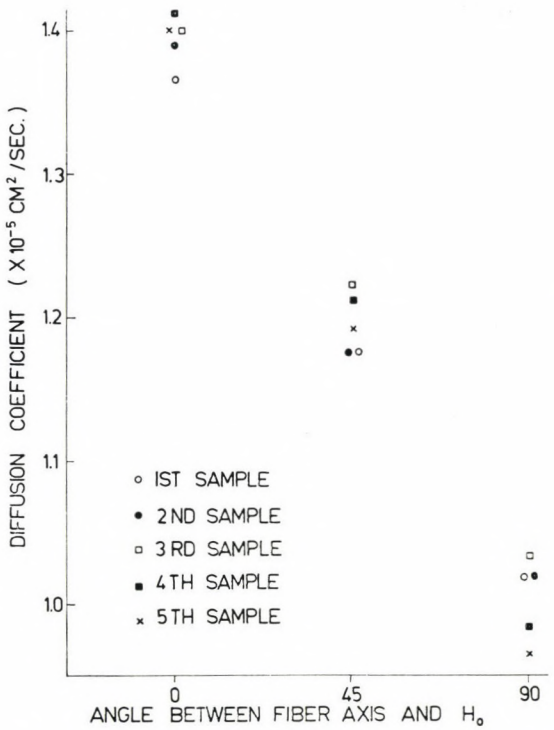

Fig. 1. The spin diffusion coefficient for oriented muscle fibers as a function of the angle between the fiber axis and the external magnetic field, H_0. The spin echo measurement gives the value of D_s along H_0. Reproduced with permission (58)

sive measurements on the same piece of muscle for which the fiber orientation had been thoroughly disordered by cutting the muscle into several pieces. This furnishes a point of reference as to what values might be expected for typical samples for which no care is taken to preserve fiber orientation.

The average value of $D_s(90)/D_s(0)$ in this study was 0.72 which represents an anisotropy of 28 per cent. The value of 2.28×10^{-5} cm^2/sec for the self diffusion coefficient of pure bulk water compares favorably with other values in the literature. Comparing the average diffusion coefficient for each fiber orientation to the value of D_s for pure bulk water (D_0), one finds

$$\frac{D_s(90)}{D_0} = 0.44, \frac{D_s(45)}{D_0} = 0.53, \text{ and } \frac{D_s(0)}{D_0} = 0.61.$$

Thus, the average diffusion coefficient measured parallel to the fiber axis is still about 40 per cent lower than D_0.

Numerous laboratories have reported that the diffusion coefficient of water molecules in living tissue is reduced by a factor of approximately two compared to pure water (Reizin et al., 1973; Caille, Hinke, 1974; Cooper et al., 1974). Three explanations for the observed reduction in the diffusion coefficient have been offered.

1. The intracellular membrane systems serve to compartmentalize the cytoplasmic water.

2. Intracellular protein structures serve as obstructions to diffusion of cytoplasmic water.

3. The water-protein interaction induces long-range changes in the water-water interaction in a substantial fraction of the intracellular water.

The possibility that the various membrane systems might serve as impenetrable barriers to diffusion was one of the first explanations offered to explain the NMR observations. The reduction of D_s due to compartment effects has been examined in considerable theoretical detail by Robertson (1966) and tested in a model system by Wayne and Cotts (1966). Chang et al. experimentally evaluated this mechanism in the skeletal muscle system and concluded that less than 10 per cent of the observed reduction could be attributed to compartment effects.

It has been proposed that because of the compartmentalization effect (interpretation 1 above), the observed diffusion will approach that of bulk water when the observation time t approaches zero. Cooper et al. have measured the spin diffusion coefficient in several tissues as a function of the observation time using the technique of pulsed field gradient spin echo. The measured diffusion coefficients at very short t did not approach the pure water value as would be expected if interpretation 1 is correct. Tanner (1975) also using the pulsed-field-gradient-spin-echo technique, has extended the measurement to times sufficiently short that the diffusion length is on the order of 1μ or less. His results confirm the observations of Cooper et al. and give a value $\frac{D_s}{D_0} = 0.4$ to 0.7. The inter-

pretation that the intracellular membrane system serves to compartmentalize the cytoplasmic water therefore is not supported by experimental evidence.

The second interpretation (i.e. obstruction of diffusion by intracellular proteins) can be tested by examining the results of this study and comparing them with the predictions of the obstruction theories. Let us examine the obstruction effect in two aspects:

(a) Can the obstruction of the non-filament like proteins account for the reduction of the spin diffusion coefficient along the direction parallel to the muscle fiber?

(b) Can the actin-myosin filaments account for the observed anisotropy of the spin diffusion of cellular water?

A model often considered to explain the reduction of D due to the obstruction effect in biological systems is that proposed by Wang (1954). This model was intended to apply to capillary flow measurements of D in dilute protein solutions. Wang's model utilizes the steady state solution of the diffusion equation and assumes that the protein molecules are stationary impenetrable ellipsoids. The general expression for the effective self diffusion coefficient D in a given direction is given by

$$D_i = (1 - \alpha_i \Phi) D_0 \qquad i = \text{a, b, c}$$

where D_0 is the self diffusion coefficient of the "free" solution, Φ is the volume fraction occupied by the hydrate protein molecules and α_i is a dimensionless numerical coefficient for diffusion parallel to the i^{th} axis of the ellipsoid. The value of α_i is determined by the dimensions of the principal semi-axes a, b and c of the ellipsoids. The equation shows that the measured diffusion coefficient D should be less than D_0 if Φ is not negligibly small. In addition, the diffusion of cell water is further reduced if the water molecules are in rapid exchange with a hydration fraction (the "direct hydration" effect). Wang shows that this effect introduces an additional factor $D' = D(1 - f)$, where f is the fraction of the water bound to the proteins. The final result for Wang's theory is

$$D_i' = (1 - \alpha_i \Phi)(1 - f) D_0 \tag{1}$$

A second approach to the problem of reduction of the measured diffusion coefficient perpendicular to the actin-myosin filament network was recently presented by Rorschach et al. In this model, the filaments are approximated by a hexagonal array of impenetrable rods of uniform radius a and lattice spacing $2R$ (see Figure 2). The effective self diffusion coefficient D_s is derived from an approximate solution to the steady state diffusion equation with the appropriate boundary conditions within a hexagonal unit cell. This calculation yields the following expressions for the spin diffusion coefficient D_s:

Parallel to fiber axes: $D_s(0) = D_0$ (2)

Perpendicular to fiber axes: $D_s(90) = \dfrac{D_0}{1 + 0.80}$ (3)

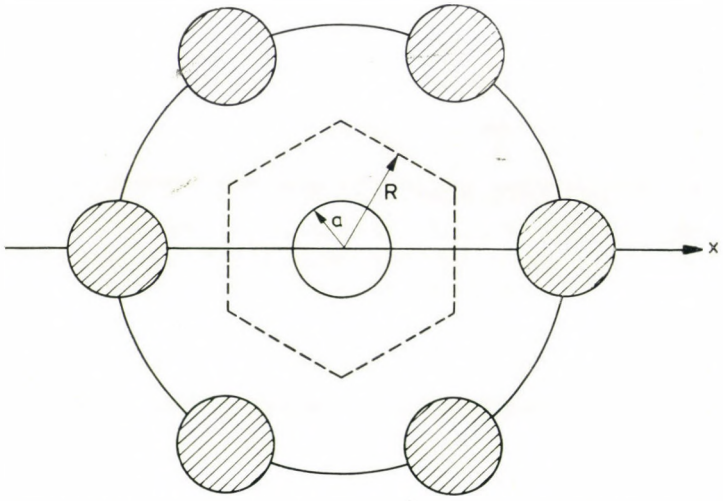

Fig. 2. A cross-sectional view of the hexagonal lattice of protein filaments. The basic hexagonal cell is shown dotted. For the purposes of the diffusion calculation, the actin and myosin filaments are taken to be cylinders of equal radius a. Reproduced with permission (58)

where D_0 is the self diffusion coefficient of the solution in the absence of obstructions, and D_s is the volume fraction of the protein.

The reduction in D_s in a cubical array of spheres has also been calculated. The results show that the measured spin diffusion coefficient is:

$$D_s = \frac{D_0}{1 + 0.63\,\Phi} \tag{4}$$

These results differ from those given by Wang for two reasons:

(a) The boundary conditions for Wang's calculation are imposed at infinity, and the flow pattern for a random array is determined by superposition. In the present calculation, the influence of the neighbors on the flow is taken into account by imposing the boundary conditions at the surface of the hexagonal cells.

(b) The diffusion in a protein solution in a capillary is influenced by the distortion of the flow and also by a geometrical factor that would reduce the transport cross-section, even if all the protein were to be congealed on the walls of the capillary. This geometrical factor does not enter in the spin echo case.

Both of these effects lead to corrections to the relation between D_s and D_0 which are of order Φ.

The experimental results presented here clearly show that the measured spin diffusion coefficient perpendicular to the muscle fiber is less than that parallel to the muscle fiber. This proves that the array of the actin-myosin filaments is an effective diffusion barrier; however, the spin diffusion coefficient parallel to

the muscle fiber is still significantly reduced from that of pure bulk water. According to Rorschach's calculation, this 40 per cent reduction of diffusion coefficient cannot be accounted for by the protein filaments, since D_s should equal to D_0 for diffusion parallel to the muscle fiber. The question then is: Can the obstruction effect of the non-filament proteins account for this reduction of diffusion?

The obstruction effect of the non-filament proteins can also be estimated from Rorschach's calculation. If we assume that these proteins are spherical, the obstruction effect can be estimated from equation (4). In order to make $\frac{D_s(0)}{D_0} = 0.61$, a value for Φ of approximately 1 is required, which obviously is too large. The cytoplasm cannot be completely filled with proteins. The alternative is to consider the "direct hydration" effect. The "direct hydration" effect introduces a reduction factor $(1 - f)$ to the measured diffusion coefficient. (This is true in both Wang's calculation and Rorschach's calculation.) Adding this factor to equation (4), the obstruction effect may be more realistically evaluated. If one assumes the non-filament proteins are dilute ($\Phi \simeq 0$), then $f \simeq 0.39$, i.e. 39 per cent of the cytoplasmic water would be hydration water. If one assumes a more reasonable value for (e.g. the non-filament proteins occupy $\simeq 5$ per cent of the volume of cytoplasm, and $\Phi = 0.05$), f will be about 0.37. The hydration water would have to constitute 37 per cent of the cellular water.

It becomes clear that the obstruction effect of protein filaments and non-filaments proteins is not sufficient to account for the reduction in the spin diffusion coefficient. The assumption that the cell water diffuses like free water and that the observed reduction in diffusion coefficient is caused by the "direct hydration" effect requires a large fraction of the cytoplasmic water to be tightly bound with the macromolecules.

Furthermore, the large degree of anisotropy in the spin diffusion coefficient also strongly indicates the inadequacy of the obstruction hypothesis. The anisotropy predicted from the geometrical dimensions of the actin-myosin system ($\Phi = 0.16$) is $\frac{D_s(0)}{D_s(90)} = 1.13$. In order to explain the measured value $\frac{D_s(0)}{D_s(90)} = 1.39$, a value of $\Phi = 0.49$ is required. This would also imply an anormous sheath of bound water. Therefore, the observed reduction and anisotropy in D_s requires either a change in the bulk diffusion coefficient of the cellular water, or an effective increase in myosin-actin filament size by a substantial sheath of bound water.

In conclusion, we have clearly demonstrated in this study that there is an anisotropy in the spin diffusion coefficient of water in muscle This anisotropy is on the order of 25 per cent to 30 per cent, and this indicates that the actin-myosin filaments system is an effective barrier Thus, for a randomly oriented sample, only about 15 per cent of the average reduction in D_s can be attributed to obstruction by the filament lattice. The fact that the value measured for $D_s(0)$ was 40 per cent less than the diffusion coefficient for pure bulk water is significant. If one analyzes the date with a calculation valid for the spin echo measurements, it is

found that the obstruction effect of the biopolymers cannot explain the reduction of the spin diffusion of water in biological systems. Either a large fraction of hydration water must be assumed to account for the observed data or that long range forces of some type are influencing the motional freedom of virtually all of the cytoplasmic water molecules. If the hydration water is bound water, it certainly is not irrotationally bound. That is, considerable motional freedom resides in the water molecules otherwise we could not "see these molecules" by NMR. Perhaps there exists domains of order to water molecules within the cell. In any event, it seems safe to conclude that cellular water cannot be considered to be equivalent to pure water.

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References

Abetsedarakaya, L. A., Miftakhutdinova, F. G., Fedetov, V. D. (1968) Biofizika, *13* 630 (Russian)—English translation, Biophysics, *13* 750
Belton, P. S., Jackson, R. R., Packer, K. J. (1972) Biochim. Biophys. Acta *286* 16
Berendsen, H. J. C. (1962) J. Chem. Phys. *36* 3297
Blanchard, K. C. (1940) Cold Spring Harbour Symp. Quant. Biol. *8* 1
Bratton, C. G., Hopkins, A. L., Weinberg, J. W. (1965) Science *147* 738
Caille, J. P., Hinke, J. A. M. (1974) Can. J. Physiol. Pharmacol. *52* 814
Chang, D. C., Hazlewood, C. F. (1975) J. Mag. Res. *18* 550
Chang, D. C., Hazlewood, C. F., Nichols, B. L., Rorschach, H. E. (1972) Nature *235* 170
Chang, D. C., Rorschach, H. E., Nichols, B. L., Hazlewood, C. F. (1973) Ann. N. Y. Acad. Sci. *204* 434
Chapman, G., McLaughlin, K. A. (1967) Nature *215* 391
Civan, M. M., Shporer, M. (1972) Biophys. J. *12* 404
Civan, M. M., Shporer, M. (1974) Biochim. Biophys. Acta *343* 399
Civan, M. M., Shporer, M. (1975) Biophys. J. *15* 299
Cleveland, G. G., Chang, D. C., Hazlewood, C. F., Rorschach H. E. (1976) Biophys. J., in press (publication scheduled for September)
Cleveland, G. G., Chang, D. C., Rorschach, H. E., Woessner, D. E., Hazlewood, C. F. (1973) Fed. Proc. *32* 302
Cope, F. W. (1969) Biophys. J. *9* 303
Cooke, R., Kuntz, I. D. (1974) Ann. Rev. Biophys. and Bioeng. *3* 95
Cooke, R., Wein, R. (1971) Biophys. J. *11* 1002
Cooke, R., Wein, R. (1973) Ann. N. Y. Acad. Sci. *204* 197
Cooper, R. L., Chang, D. B., Young, A. C., Martin, C. J., Ancker-Johnson, B. (1974) Biophys. J. *14* 161
Damadian, R. (1971) Science *171* 1151
Eisenberg, D., Kauzman, W. (1969) The Structure and Properties of Water, Oxford University Press, New York

Ernst, E. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. *5* 57
Ernst, E., Fricker, J. (1934) Pflügers Arch. Ges. Physiol. *234* 360
Ernst, E., Tigyi, J., Zahorcsek, A. (1950) Acta Physiol. Acad. Sci. Hung. *1* 11
Finch, E. D., Harman, J. F., Muller, B. H. (1971) Arch. Biochem. Biophys. *147* 299
Fritz, O. G., Swift, T. J. (1967) Biophys. J. *7* 675
Fung, B. M., McGaughy, T. W. (1974) Biochim. Biophys. Acta *343* 663
Gortner, R. A., Gortner, W. A. (1934) J. Gen. Physiol. *17* 327
Hansen, J. R. (1971) Biochim. Biophys. Acta *230* 482
Hazlewood, C. F., Chang, D. C., Nichols, B. L., Woessner, D. E. (1974) Biophys. J. *14* 583
Hazlewood, C. F., Cleveland, G., Medina, D. (1974) J. Nat. Cancer Inst. *52* 1849
Hazlewood, C. F., Chang, D. C., Medina, D., Cleveland, G., Nichols, B. L. (1972) Proc. Nat. Acad. Sci. *69* 1478
Hazlewood, C. F., Chang, D. C., Nichols, B. L., Rorschach, H. E. (1971) J. Mol. Cell Card. *2* 51
Hazlewood, C. F., Nichols, B. L., Chamberlain, N. F. (1969) Nature *222* 747
Hechter, O. (1965) Annals New York Acad. Sci. *125* 625
Hill, A. V. (1930) Proc. Roy. Soc. (London), Ser. 13, *106* 477
Hodgkin, A. L. (1961) Biol. Rev. *26* 339
Hodgkin, A. L., Huxley, A. F. (1939) Nature *144* 710
Katz, B. (1966) Nerve, Muscle and Synapse, McGraw-Hill, New York.
Ling, G. N. (1962) Physical Theory of the Living State, Blaisdell, Philadelphia
Ling, G. N. (1965) Annals New York Acad. Sci. *125* 401
Ling, G. N. (1969) Intern. Rev. Cytol. *26* 1
Ling, G. N. (1972) in Water and Aqueous Solutions (Horne, R. A. editor) Wiley Interscience, New York, pages 663—700
Neville, M. C., Paterson, C. A., Pae, J. L., Woessner, D. E. (1974) Science *184* 1072
Outhred, R. K., George, E. P. (1973) Biophys. J. *13* 97
Pearson, R. T., Duff, I. D., Derbyshire, W., Blanchard, J. M. F. (1974) Biochim. Biophys. Acta *362* 188
Reisin, I. L., Ling, G. N. (1973) Physiol. Chem. and Physics *4* 183
Robertson, B. (1966) Phys. Rev. *151* 273
Rorschach, H. E., Chang, D. C., Hazlewood, C. F., Nichols, B. L. (1973) Annals New York Acad. Sci. *204* 444
Rubner, M. (1922) Abhandl. Preuss. Akad. Wiss. Phys. Math. K1 *1* 1
Swift, T. J., Fritz, O. G. (1969) Biophys. J. *9* 54
Tanner, J. E. (1975) Self-diffusion in cells and tissues. Office of Naval Research, Report NWSC/CR/RDTR-6 Division of Medical and Dental Science, Arlington, Virginia 22217
Thoenes, F. (1925) Biochem. Z. *157* 174
Troshin, A. B. (1966) Problems of Cell Permeability, Pergamon Press, New York
Walter, J. A., Hope, A. B. (1971) Prog. Biophys. and Molec. Biol. *23* 1
Wang, J. H. (1954) J. Am. Chem. Soc. *76* 4755
Wayner, R. C., Cotts, R. M. (1966) Phys. Rev. *151* 264
Zimmerman, J. R., Brittin, W. E. (1957) J. Phys. Chem. *61* 1328

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Bound Water* its Real Meaning

By

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After Professor Hazlewood's valuable and interesting lecture permit me, please, to make a short note to the problem of the real meaning of "bound water" encountered so often in the literature. Figure 1 shows the values of the vapour pressure of water (in Hgmm) as a function of temperature; the first curve demonstrates the values for clean water; the second that of a solution of 0.25 M $CuSO_4$ computed approximately on the supposition that it dissociates totally; consequently the relative lowering of the vapour pressure comes to $\frac{p_1}{p_0} \sim 1-0.01 = 0.99$ according to Roault's formula. These values are represented by dots and are very close to those for clean water.

The next curve (3) shows computed values of a $CuSO_4$ solution of concentration 10 times greater than that of the 0.25 molal solution; in this case the formula $\frac{p_1}{p_0} \sim 1-0.1 = 0.90$ could be valid. The 4th curve belongs to the crystal $CuSO_4 \cdot 5\ H_2O$, the 5th to $CuSO_4 \cdot 3\ H_2O$ (and the last one to $CuSO_4 \cdot H_2O$).

These curves have the appearence as if there were a continuous transition between the changes of the relative lowering of vapour pressure as a function of water content; that is to say, if there were a continuous transition between the degrees of boundedness of the water in the systems discussed above.

That is, however, in contrast to the real situation. Figure 2 shows that the vapour pressure of the crystal $CuSO_4 \cdot 5\ H_2O$ comes to 47 Hgmm at 50 °C ($p_0 \sim 92.5$) and remains at this value during continuous decrease of the water content. It drops, however, immediately and abruptly to 30 Hgmm, when the new state of the crystal $CuSO_4 \cdot 3\ H_2O$ is arrived at. Similarly, this value lasts as long as the water content decreases further till the new state: the crystal of $CuSO_4 \cdot H_2O$ appears showing the vapour pressure of 1 Hgmm. Thus the vapour pressure of these crystals of $CuSO_4$ changes *discontinuously* according to the water contents of 0.56, 0.34 and 0.11 g water per g $CuSO_4$. These values can be considered the amount of bound water in these crystals.

* Comments made on the occasion of Professor Hazlewood's preceding lecture.

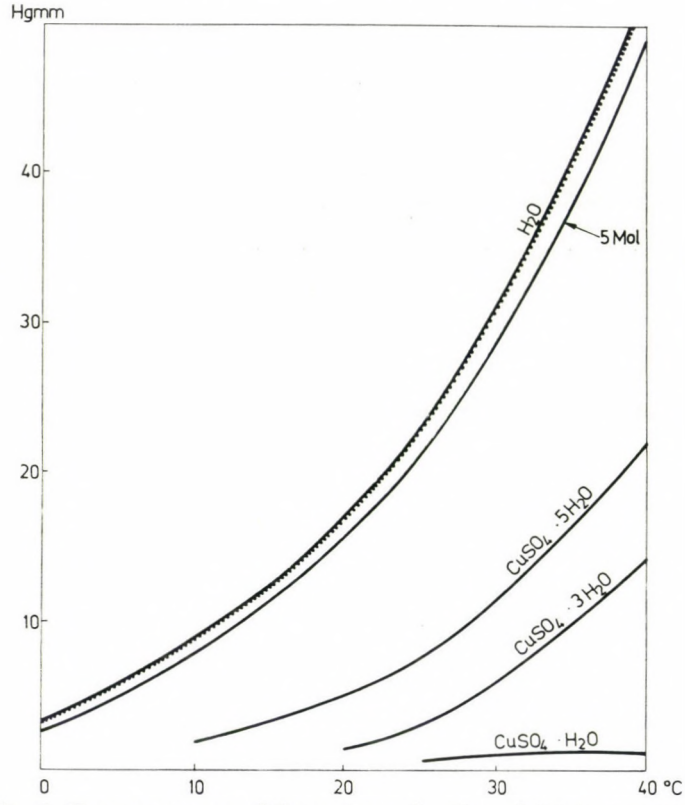

Fig. 1. Vapour pressure of the water as function of the temperature

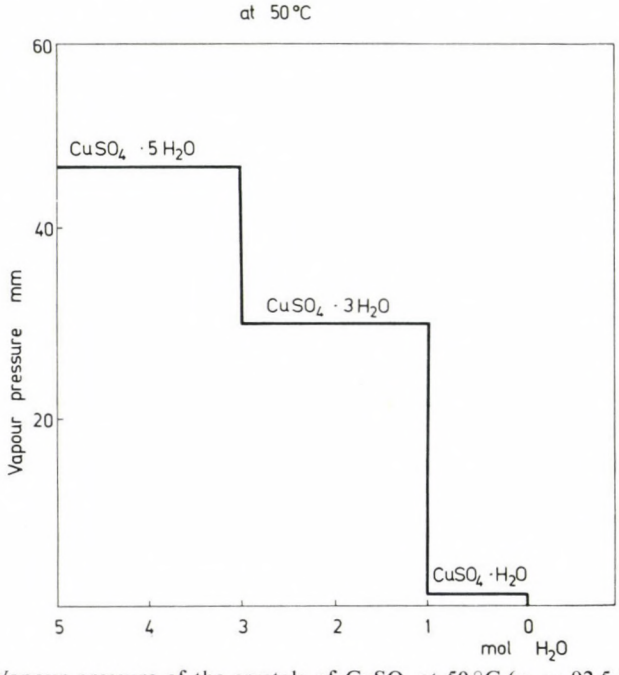

Fig. 2. Vapour pressure of the crystals of $CuSO_4$ at 50 °C ($p_0 \sim 92.5$ Hgmm)

Dealing with the question of bound water in biological systems, I wish to mention the question concerning the possible different ways (and degrees) of bonding the water: solution, hydration, adsorption, swelling, chemical compound, complex compound etc. Especially I want a) to query the statements encountered in many publications according to which some biological compounds would have a single value of bound water content of e.g. 0.3 g/g protein, and b) to raise the question whether there are similar gaps in the values of bound water also in biological systems.

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Reverberating Excitation of Nerve-Muscle Preparations

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Experiments were performed on excitatory circuits constructed from four, three, two and one sciatic-gastrocnemius preparations, respectively. The nerve-muscle preparations which normally responded to a single stimulus with only one action potential produced a long-lasting excitation when arranged into special structures quasi-modelling a reverberating process.

Introduction

It has been known since the last century that a muscle can excite a nerve in contact with it (Matteucci, 1842). Later on, the excitatory effect of muscle activity on a nerve was demonstrated in various forms, such as the burst activity of the ventral root (Masland, Wigton, 1940), the "secondary centripetal discharge" of the ventral root (Eccles et al., 1942), the "back response" recorded from the ventral root (Leksell, 1945; Brown, Matthews, 1960), the "early response" of the muscle spindle (Granit et al., 1959), the antidromic activity of the motor nerve (Werner, 1961) and the re-excitation of the muscle (Epstein, Jackson, 1970). The results of these experiments showed that muscle activity could generate nerve excitations within the muscles. However, experiments performed on frog's preparations demonstrated that the muscles could stimulate the nerve which was surrounded with muscles or which was situated on a muscle also under in situ conditions (Biró, 1975; Biró, Vu-Duy-Thinh, 1977).

Taking the Matteucci's experiment as a starting point of investigation, we have established that excitation could be trasmitted through 4 to 6 nerve-muscle preparations (Biró, 1975). On the basis of this finding it arises the question of what will happen to the excitation if a circuit is constructed from the nerve-muscle preparations. In this case it would be possible that a long-lasting excitation could develop on the preparations due to the circulation of excitation.

It has been the aim of the present work to investigate the activity of different circuits constructed from nerve-muscle preparations.

Methods

The experiments were performed on isolated sciatic-gastrocnemius preparations of the frog (Rana esculenta). The preparations were connected to each other by means of their nerves laying in favourable position on the muscle of another preparation. These connections allowed the construction of different circuits containing four, three or two preparations, respectively. In some experiments only one preparation was used and, in this case, the nerve was folded and placed on its own muscle.

The responses of the preparations were elicited with a single square-wave stimulus 2 V in amplitude and 0.1 ms in duration applied to the distal portion of one of the nerves. The proximal part of the stimulated nerve was laid on that muscle from which the action potentials were recorded. The pairs of the electrode used for stimulation and recording were made from platinum wire 1 mm in diameter. The connections between the electrodes and the preparations are shown in the Figures.

The electrical activity of the muscle was recorded by oscillographic method. In some experiments an analogous tape recorder was also used for recording action potentials.

The experiments were carried out at room temperature (22–24 °C); care was taken to keep the preparations under wet conditions.

Results

In the first series of the experiments, the excitatory circuits were constructed from four preparations (Fig. 1.a.). After stimulation of one of the nerves with a single square-wave impulse a series of action potentials could be recorded from the muscle on which the stimulated nerve laid. Similar results were obtained also in the case of excitatory circuits containing three, two or one preparations, respectively (Fig. 1b, c, d). The comparison of the oscillograms showed that decreasing the number of preparations in the excitatory circuits decreased the latency and increased the frequency of the series of action potentials. The duration of the series of action potentials showed considerable variations in individual experiments. However, in most cases using four, three and two preparations the duration was longer than 0.5 s. In an experiment performed on three preparations, the series of the action potentials lasted 3 s. The reason of the excitatory process coming to an end is that the magnitude of the action potential produced by one of the muscles decreases below the threshold of the nerve laid on the muscle.

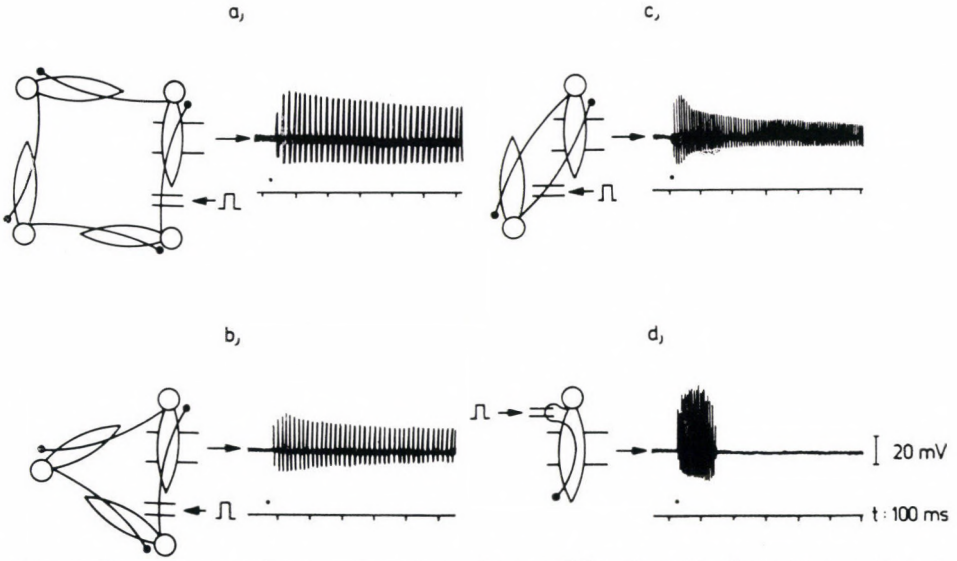

Fig. 1. Reverberation of the excitatory circuits containing four (a), three (b), two (c) and one (d) sciatic-gastrocnemius preparations. Upper beam: series of the action potentials recorded from the muscle on which the stimulated nerve laid. Lower beam: stimulatory impulse (2 V, 0.1 ms) shown as a point above time markers.

Discussion

The significance of the results demonstrated in Fig. 1 is that nerve-muscle preparations normally responding to a single stimulus with one action potentials only can also produce a long-lasting excitation when arranged into special structures.

The excitatory activity of the circuits constructed from these preparations reminds of the reverberation of excitation (Lorente de Nó, 1938; Hebb, 1949; Burns, 1954; Verzeano, Negishi, 1960; Konorski, 1961; John, 1967). In the case of single preparations the tetanic response of the muscle supports the experimental results, according to which repetitive excitations can be generated by the stimulatory effect of the muscle activity on its own motor nerve (Masland, Wigton, 1940; Epstein, Jackson, 1970).

Further investigations with this type of constructions made from nerve-muscle preparations could serve for modelling various excitatory phenomena.

References

Biró, G. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. *10* 287
Biró, G., Vu-Duy-Thinh (1977) Acta Biochim. Biophys. Acad. Sci. Hung. *12* 57
Brown, M. C., Matthews, P. B. C. (1960) J. Physiol. *150* 332

Burns, B. D. (1954) J. Physiol. *125* 427
Eccles, J. C., Katz, B., Kuffler, S. W. (1942) J. Neurophysiol. *5* 211
Epstein, R. A., Jackson, S. H. (1970) J. Appl. Physiol. *28* 407
Granit, R., Pompeiano, O., Waltman, B. (1959) J. Physiol. *147* 399
Hebb, D. O. (1949) The Organization of Behaviour, John Wiley and Sons, Inc., New York
John, E. R. (1967) Mechanisms of Memory, Academic Press, New York
Konorski, J. (1961) in: Brain Mechanisms and Learning, Ed.: J. F. Delafresnaye, Blackwell Scientific Publications, Oxford, p. 115
Leksell, L. (1945) Acta Physiol. Scand. *10* Suppl. XXXI.
Lorente de Nó, R. (1938) J. Neurophysiol. *1* 207
Masland, R. L., Wigton, R. S. (1940) J. Neurophysiol. *5* 153
Matteucci, C. (1842) cited in: Handbook of Physiology Ed.: J. Field, Section 1: Neurophysiology, American Physiological Society, Washington, D. C. 1959. Vol. 1. p. 20
Verzeano, M., Negishi, K. (1960) J. Gen. Physiol. *43* Suppl. 177
Werner, G. (1961) J. Neurophysiol. *24* 401

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The Role of Shortening and Tension in the Heatproduction of Muscle

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The dependence of the heatproduction of muscle on shortening was examined. An inverse relation was found between heatproduction and shortening as well as tension and shortening, while there was a close positive correlation between heatproduction and tension. The results emphasize the primary importance of tension as compared in the heat production of muscle, to shortening.

Introduction

It was written by Hill (1938) in his summarizing work that in a muscle shortening during a tetanically maintained contraction extra energy is released in two forms (as compared to isometric tension): a) as "shortening heat" proportional with shortening, b) as mechanical work. The shortening heat does not depend on loading, speed and work. Similarly Abbott (1951) stated that shortening heat is proportional with shortening in a wide range. Aubert (1952) found that extra heatproduction is greater with a smaller speed of shortening. In his later work Hill (1953) measured on extra heatproduction proportional with work (i.e. loading) in case of shortenings of the same degree. This caused him to revise completely the conception of shortening heat (Hill, 1964a). He acknowledged the dependence of shortening heat on speed and loading. He attributed the almost 30 per cent deviation from former results in 1938 to the methods.

Several experiments and ideas have originated from our laboratory, which have called the attention to a possible error of the methods in shortening heat measurement (Tigyi, 1959; Ernst, 1963). Chapman and Gibbs (1972a, 1972b) as well as Lőrinczi (1974) came to conclusions similar to those of Tigyi's and Ernst's.

In the opinion of Aubert and Lebacq (1971) shortening heat is well measurable if it is continuously examined up to the end of relaxation. This lead to the conclusion that shortening heat cannot be a part of a reversible cyclic process during relaxation, but it must come from some chemical source of energy.

In the explanation of Homsher and Mommaerts (1973) the cause of the difference between shortening heat in the cases of tetanus and twitch is that Hill's definition of shortening heat (SH) is inaccurate. They say this difference can be

eliminated by finding the muscle lengths belonging to the same force in the length-force curve, and letting the muscle shorten between these two lengths of muscle at a constant tension in such a way, that the twitch or tetanus becomes isometric in the end. Taking the heatproduction of isometric twitch or tetanus of the same force as a basis, the difference between the two heatproductions provides the so-called force-determined shortening heat (SHF).

In our former work (Lőrinczi, Tigyi, 1976) we found that the length dependence of the heatproduction of muscle calls the attention to the decisive role of tension in contrast to the shortening. Our experimental result according to which $Q_{\text{isometr.}} > Q_{\text{isoton.}}$ at every muscle length – entirely contradicts to shortening heat. (In Hill's opinion [1949] shortening heat is independent from muscle length.)

The contradicting data in the literature encouraged us to reinvestigate the role of shortening in heat production.

Methods

M. semimembranosus excised from *Rana esculenta* was used as experimental object. The measurements were performed at room temperature (20 °C). The muscle was kept in normal Ringer's solution from excision until placing into the calorimeter. A super-maximum (6 V) square-pulse of 10 ms was used as a stimulus, and the muscle was brought in the state of a 66 Hz smooth tetanus for 2 s.

Two procedures were employed for the study. In the first the rate of shortening was adjusted with an isometric stop. The muscle contracted from resting length (l_0) with increasing shortening of every mm between 0–9 mm. During the selected shortening the muscle moved isotonically against the load necessary for the adjustment of resting length (7 ponds), after that contraction became isometric, the tension of which was measured with a capacitive force-transducer. In the second series of measurements the muscles shortened without tension from resting length (l_0) as well as excised length (about 0.9 l_0) until 0.7 l_0, and from this point the responses became strictly isometric. The heatproduction was measured with the *thermophile heat conduction* microcalorimeter developed in our institute (Lőrinczi, Futó, 1974).

Results

Figure 1a shows the dependence of the heatproduction of the muscle on shortening in our first series of experiments. It can be seen that heatproduction decreases when shortening increases.

In Figure 1b the tension of the same contractions is plotted against shortening. The values on the diagrams are the results of the measurements performed on seven semimembranosus muscles (at every shortening) with a Hewlett-Packard calculator.

Figure 2a shows the difference in heatproduction between the contraction with shortening (isotonic) and the contraction with tension (isometric) at resting length which was used as a basis. It can be seen that the heatproduction steeply decreases with the increasing rate of shortening i.e. with decreasing tension.

In Fig. 2b the curve denoted with A shows the decrease of the heatproduction originating from the length-dependence of heatproduction (calculated from our previous work. Lőrinczi, Tigyi, 1976). curve B is the "shortening heat" calculated on the basis of Hill's work (1938), curve C shows the resultant of the two processes,

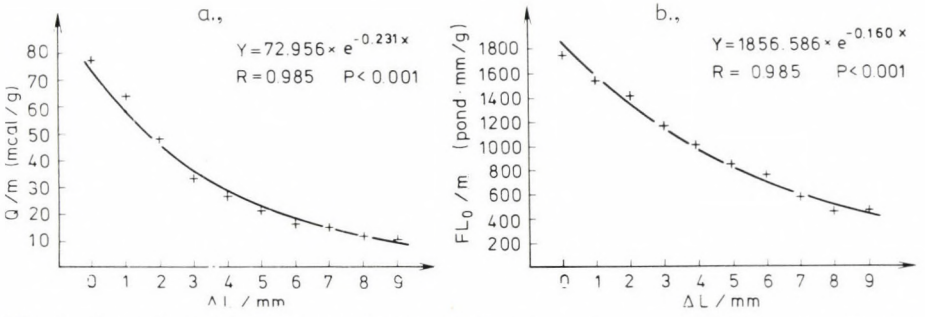

Fig. 1a. Dependence of the heatproduction of muscle on shortening. The curve is a Hewlett-Packard's calculator-fitting for the data of seven muscles measured at every shortening. (R= = correlation coefficient, $Y = Q/m$, the heatproduction normalized for mass and $x = \Delta l$, where Δl is the shortening, $P < 0.001$ is level the of probability

Fig. 1b. Force development (F) of the preceding (Fig. 1a) contractions normalized for resting length and mass plotted against shortening. (Symbols: $Y = Fl_0/m$, l_0 is the resting length, the others are the same as the ones in Fig. 1a)

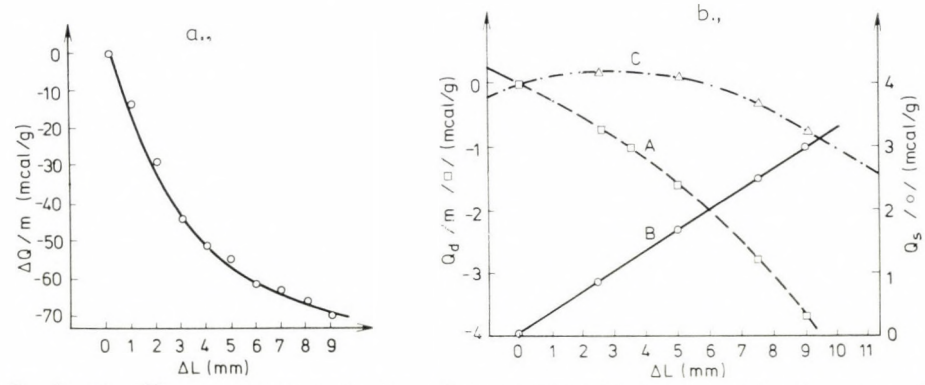

Fig. 2a. The difference in heatproduction of contractions with shortening (but then stopped) and contractions with tension at resting length plotted against shortening

Fig. 2b. *A:* Decrease of heatproduction calculated from the length dependence of heatproduction plotted against shortening (see the test) (□). *B:* Shortening heat calculated on the basis of Hill's results (o). *C:* Resultant of the two processes (see it in details in the text) (Δ)

from which it can be seen that the resultant heatproduction (if there exists a shortening heat) would have an extremum in the function of shortening.

With the second series of measurements our aim was to create almost equal starting and finishing states for the muscle. In the first case the muscles shortened from resting length (l_0) to 0.7 l_0 against 7 ponds, in the second case from the excised length ($\sim 0.9\ l_0$) to 0.7 l_0. The difference in the starting tension is 1 to 1.5 per cent of the maximum tension measured at resting length. The tension of the finishing state is the same. But macroscopic shortening is about 1/3 greater in the first case. In our measurements (Table 1) no significant difference was experienced

Table 1

Heat production of muscle for tetani starting from excised-, and resting lengths

	From excised length to 0.7 l_0					From resting length (l_0) to 0.7 l_0					
m=1180 mg l_0=36 mm	1080 36	1160 37	1230 37	1050 30	1090 37	1050 37	1210 37	1250 37	1040 36	1120 36	1120 37
	Q/m mcal/g					Q/m mcal/g					
30.33	32.72	30.17	31.36	30.84	29.92	30.08	25.71	32.21	27.89	31.48	31.76
28.13	27.67	27.08	26.94	28.32	28.47	28.45	25.33	29.03	26.44	29.18	29.12
27.20	24.08	24.76	23.85	26.97	27.33	27.13	25.16	27.63	25.11	27.34	27.48
26.20	22.65	23.09	21.06	25.82	26.08	25.72	24.48	24.42	24.83	25.09	25.02
25.58	21.51	21.41	19.83	24.03	25.42	24.99	24.04	21.04	24.16	23.62	22.11
25.31	20.32	19.54	18.76	23.91	25.17	23.74	22.69	20.61	22.66	21.87	20.36
	Q/m average = 25.61 mcal/g Stand. dev. = 3.54 mcal/g					25.78 3.08					

between the heatproduction of the two cases when $p < 0.01$, and this experience was not influenced by the seasonal effect. (6 measurements for each muscle in 6 pairs of muscles, evaluation with a Hewlett-Packard calculator, corrected for the fatigue effect.)

Discussion

Hill measured the shortening heat in his original work (1938) and in a similar one (1964a) by using an isometric tetanus as a basis. When the muscle reached its maximum tension, he suddenly released the muscle, to let it shorten isotonically then the contraction became isometric again at a smaller length than the starting length corresponding to the shortening and naturally with a smaller tension. The shortening heat was provided by the difference in heatproduction of the original isometric contraction (which was used as a so called "physiological base

line") and this latter released contraction (isometric tension – shortening – smaller isometric tension).

It is known from classic works, that the heatproduction of muscle greatly depends on its preceding state. Isotonic contraction is a contraction at a *constant* tension and not a shortening during a contraction at a tension decreased from a maximum tension.

Isotonia is transgressed several times because there are several processes which cannot even be estimated, the heatproduction of the process when the original isometric tetanus becomes "isotonic" cannot be traced. It is also unknown, to what extent the process of the isotonic becoming isometric again affected Hill's measurements. (Because Hill based his estimations on the fact that the effect originating from the length-dependence of heatproduction is rather small (Hill, 1938, 1958).)

Therefore we chose the method of comparing the heatproduction of contractions becoming isometric after different shortening. This method offers a better possibility for the estimation of the contribution of length dependence of the transition from isotonia to isometria and heatproduction if shortening heat really exists.

It is known from the measurements of Woledge (1961), that the decrease of tension is accompanied by heatproduction and its increase is accompanied by heat absorption in an active muscle. He gained a relationship $Q = -0.014\, l_0 \Delta P$ for the degree of heatproduction, where l_0 is the resting length, ΔP is the change of tension. Examining the original work of Hill (1938) $\Delta P \sim 30-90$ per cent. This means that the heat produced by thermoelastic effect is in the order of magnitude of the shortening heat.

According to Hill (1964b) the shortening heat is the longest and the "positive feed back" caused by tension is the smallest at small loading. This is why we chose small loading. The heat absorption described by Woledge (1961) ought to be the greatest at smaller shortenings and the smallest at maximum shortenings. In our experiments contraction which was stopped after a shortening of 1 mm was one from a tension of near zero to an almost maximum tension, while after shortening of 9 mm-s tension was only 8 per cent of the 1 mm case. Thus we ought to have experienced the greatest heatproduction here in the case of the shortening of 9 mm-s, if there existed a shortening heat, and heatproduction would be greater in a (isotonic) contraction with shortening than in a (isometric) one with tension.

As it was not possible to discover the chemical reaction responsible for shortening in biochemical experiments (Mommaerts et al., 1962; Carlson et al., 1963; Kushmerick et al., 1969), we should be more critical in the treatment of shortening heat as indicated by the above results. The problem is made more complicated by the fact that the same working-team came to opposing conclusions (Davies, 1963; Davies et al., 1967) and there is a contradiction between the individual authors too (Davies et al., 1967; Aubert, Lebacq, 1971: Lebacq, 1972, Dickinson et al., 1974). Therefore the result of Curtin and Woledge (1975) is

very remarkable, according to which heat + work after tetani of 2 and 5 s is significantly greater then the value calculated from chemical changes. They try to explain the difference with the change in entropy of the occurring conformational change.

Homsher and Mommaerts (1973) as well as Homsher, Mommaerts, Ricchiuti (1973) consider Hill's definition of shortening heat is inaccurate.

The basis of their work is the work of Homsher, Mommaerts, Ricchiuti, Wallner (1972). They suppose that the creation and maintenance of tension requires the energy determined by the tension but independent from the type of contraction. This is the basis of their definition of force-determined shortening heat (SHF) as the difference between heatproduction of a shortening contraction and an isometric contraction of the same force development and inner work.

In the second series of our experiments a similar inner work and similar force development were found in final state ($0.7\ l_0$) and they had nearly the same starting tension, there was a difference only in their macroscopic shortening. No significant difference between the two types were found (for excised length an average of *Q/m:* 25.61 mcal/g; stand. dev.: 3.54 mcal/g; for resting length an average of *Q/m:* 25.78 mcal/g; stand. dev.: 3.08 cmal/g). The measurements of Homsher et al. were performed at 0 °C, ours at 20 °C. The measurements of Homsher et al. show that muscle produces the same activation and tension-related heat at 20 °C as at 0 °C, but it produces only half of SHF, which can account for our contradicting results.

On the basis of the above data and the results of our Institute the shortening heat could be considered as an essential part of muscle contraction only on the basis of the concordant results of methods independent from each other. But this condition is not fulfilled (Ernst, 1963).

Our present work stresses the essence of muscle tension in contraction on the one hand, which can be related to the polymer crystallization of muscle proteins according to Ernst's hypothesis. Juhász-Mórocz (1962) experienced heat-production with the passive stretch of muscle and myosin bundles, and heat absorption with the release. Her interpretation of the result was that in the case of isothermia the more ordered state setting in with stretching is accompanied by heat release, while the restoration of the original disordered state is accompanied by heat absorption, on the other hand it calls the attention to the importance of the methodical problem and to the necessity of more sensitive measurements depending on temperature.

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References

Abbott, B. C. (1951) J. Physiol. *112* 488
Aubert, X. (1952) J. Physiol. Paris *44* 206
Aubert, X., Lebacq, J. (1971) J. Physiol. *216* 181
Carlson, F. D., Hardy, D. J., Wilkie, D. R. (1963) J. gen. Physiol. *466* 851
Chapman, J. B., Gibbs, C. L. (1972a) Biophys. J. *12* 215
Chapman, J. B., Gibbs, C. L. (1972b) Biophys. J. *12* 227
Curtin, N. A., Woledge, R. C. (1975) J. Physiol. *245* 737
Davies, R. E. (1963) Nature *199* 1068
Davies, R. E., Kushmerick, M. J., Larson, R. E. (1967) Nature *214* 148
Dickinson, V. A., Woledge, R. C. (1974) J. Physiol. *242* 98—99
Ernst, E. (1963) Biophysics of the Striated Muscle (363 p. and 373 p.) Akadémiai Kiadó Budapest
Hill, A. V. (1938) Proc. Roy. Soc. B *126* 136
Hill, A. V. (1949) Proc. Roy. Soc. B *136* 339
Hill, A. V. (1953) Proc. Roy. Soc. B *141* 503
Hill, A. V. (1958) Proc. Roy. Soc. B *149* 58
Hill, A. V. (1964a) Proc. Roy. Soc. B *159* 297
Hill, A. V. (1964b) Proc. Roy. Soc. B *159* 596
Homsher, E., Mommaerts, W. F. H. M., Ricchiuti, N. V., Wallner, A. (1972) J. Physiol. *220* 601
Homsher, E., Mommaerts, W. F. H. M. (1973) Biophys. Soc. Abstr. *13* 186a
Homsher, E., Mommaerts, W. F. H. M., Ricchiuti, N. V., (1973) J. Gen. Physiol *62* 677
Juhász-Mórocz, M. (1962) Acta Physiol. Acad. Sci. Hung. *22* 281
Kushmerick, M. J., Larson, R. E., Davies, R. E. (1969) Proc. Roy. Soc. B *174* 293
Lebacq, J. (1972) J. Physiol. *224* 141
Lőrinczi, D., Futó, Z. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 371
Lőrinczi, D. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 383
Lőrinczi, D., Tigyi, J. (1976) Acta Biochim. Biophys. Acad. Sci. Hung. *11*. 311.
Mommaerts, W. F. H. M., Seraydarian, K., Marechal, C. (1962) Biochem. Biophys. Acta *57* 1
Tigyi, J. (1959) Acta Physiol. Acad. Sci. Hung. *16* 129
Woledge, R. C. (1961) J. Physiol. *155* 187

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 12 (3), pp. 291–302 (1977)

Electrical Properties of the Cardiac Muscle Cell Membrane and its Role in the Excitation-Contraction Coupling

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During extracellular stimulation, the electrical properties of the cardiac muscle cell membrane were investigated by means of intracellular microelectrodes. The current-voltage characteristic was S-shaped, and showed non-linearity over given voltage ranges in hyperpolarizing and depolarizing directions as well. The space constant of the membrane was 1.7 ± 0.1 mm, which decreased after treatment with Ca-free or hypertonic solutions. The mean membrane time constant was 70 msec, and the input resistance was about 8 Mohm. The specific membrane resistance (R_m) and capacity (C_m) were 20 000 ohm . cm^2 and 5–6 μF/cm^2, respectively. After glycerol treatment the R_m decreased to 10 000 ohm . cm^2 and the C_m to 4–4.5 μF/cm^2. On the basis of our results it seems reasonable to suggest that the functional relationships between muscle cells might be realized through low resistance junctions, and the effectiveness of these junctions appeares to be weakened both by Ca-free solution and glycerol treatment. The specific membrane capacity was lowered by 20 per cent, after treatment with a hypertonic solution, however 60 per cent of the contractility remained unchanged. Thus, we conclude; 1. a part of the C_m originates from the infoldings of the surface membrane, and 2. the snail heart muscle cells are insensitive to glycerol treatment. Since the excitation-contraction (E-C) was not affected by the glycerol treatment it can be assumed that in snail heart the coupling of the E-C is a "direct coupling" similarly to other heart, smooth and slow muscles.

Introduction

Our knowledge of the passive electrical parameters of the molluscan heart muscle is still scanty (Irisawa et al., 1973), while several reports have been published on the ion-mechanism of transmembrane potentials, and on the effect of the polarizing current (Irisawa et al., 1961; Ebara, 1964; Irisawa et al., 1967; Ebara, Sato, 1971; Kuwasawa, Hill, 1972; Kiss, S.–Rózsa, 1973). At the same time, to know the electrical properties of the surface membrane is essential for understand the mechanism of the excitation, conduction and excitation-contraction coupling. It has been shown by Ebara (1964) in oyster, and Irisawa et al. (1973) in mussel heart that the functional connections between muscle cells similarly to heart muscle cells of vertebrates (Weidmann, 1969) originate in special membrane structures characterised by low resistance. Such membrane structures were reported by North (1963) and Elekes et al. (1973) in snail heart, too. Furthermore the influx of Ca-ions through the surface membrane in the heart muscle is frequently

supposed to play an important role in the excitation-contraction coupling. This way of activation of the contraction was reported in crayfish and amphioxus muscles as well (Gainer, 1968; Hagiwara et al., 1968; Hagiwara et al., 1971).

The aim of the present study was to examine the electrical properties of (1) the heart muscle membrane, (2) the character of the electrical coupling between muscle cells, and (3) the role of the surface membrane in the mechanism of excitation-contraction coupling.

Material and methods

The experiments were carried out on isolated snail hearts *(Helix pomatia L.)* at room temperature (22–25 °C). The volume of the chamber containing the preparation was 0.5 cm^3, and the incubating medium could be changed continuously. Electrodes: The passive and active electrical responses of the muscle membranes were registered by the aid of intracellular microelectrodes filled with 3 M KCl. A suction electrode with tip diameter of 0.2–0.3 mm was used for stimulation. A silver plate of about 0.5 cm^2 was employed as indifferent electrode. The duration of the $1-5\times10^{-8}$ A hyperpolarizing and depolarizing step current impulse was 1 sec. Simultaneously with the microelectrode used for registration, the tip of another microelectrode was also immersed into the physiological solution surrounding the preparation. If the electric properties of the two microelectrodes were almost the same the capacitive artifact could be well compensated (Bonke, 1973; Kiss, S. – Rózsa, 1975). The contractions of the heart were registrated on a kimograph. Solutions: The composition of the physiological solution was the following: NaCl 6.5 g; KCl 0.14 g; $NaHCO_3$ 0.2 g; $CaCl_2$ 0.12 g per litre of distilled water. In the Ca-free solution the $CaCl_2$ was substituted by the equivalent amounts of NaCl, besides this 3 mM EDTA was also added.

The hypertonic solutions were made by adding 400 or 800 mM glycerol. The perparations were incubated in the glycerol containing solution for 2–3 hours. Theoretical considerations:

The electrical parameters of heart muscle fibres were determined according to the square wave analysis. In the Purkinje fibre Weidmann (1952) showed that the spread of the electrotonic potentials is exponential and could be described with the cable equation. Hodgkin and Rushton (1946) described the spatial distribution and the time course of the electrotonic potentials (ETP) in the following equation:

$$V_m = \frac{r_i \lambda I_0}{4}\left\{\exp(-X)\operatorname{erfc}\frac{X}{2\sqrt{T}} - \sqrt{T} - \exp(X)\operatorname{erfc}\left(\frac{X}{2\sqrt{T}} + \sqrt{T}\right)\right\} \quad (1)$$

Where: erfc is the complementary error function

V_m = the change in the potential difference across the membrane in mV

λ = the space constant in cm
r_i = resistance per unit length of sarcoplasma ohm.cm
I_0 = applied current at the distance $x = 0$ from the stimulating electrode in A
τ_m = the membrane time constant in msec
t = time in msec
T = t/τ_m

Equation (1) describes the voltage response of infinite cable to prolonged step current change. In the steady-state when $T \to \infty$, erfc $(T) \to$ O and erfc $(-T) \to 2$, the expression in the brackets in Equation (1) is simplified and will be equal to $2e^{-x/\lambda}$, since $X = x/\lambda$. According to this the amplitude of the ETP at distance x from the stimulating electrode becomes:

$$V_x = \frac{1}{2} r_i \lambda I_0 e^{-x/\lambda} \tag{2}$$

while at distance $x = 0$ Equation (2) becomes:

$$V_0 = \frac{1}{2} r_i \lambda I_0 \tag{3}$$

where: V_0 = the amplitude of ETP at the distance x from the stimulating electrode.

From Equations (2) and (3) it follows that in the case of the square wave analysis the amplitudes of ETPs could be determined as:

$$V_x = V_0 e^{-x/\lambda} \tag{4}$$

Using the cable characteristics, the electrical characteristics of the surface membrane can be evaluated. Our calculations were made with the simplified equations proposed by Sakamoto and Goto (1971);

$$R_m = \frac{2R_i}{a} \lambda^2 \tag{5}$$

$$\tau_m = R_m C_m \tag{6}$$

where: R_m = specific resitance of the membrane (ohm cm^2)
C_m = specific membrane capacity (μF/cm^2)
a = 4×10^{-4} cm is the radius of the fibre diameter
r_i = 240 ohm.cm (Weidmann, 1952)

At the calculations several assumptions were made, namely: it was proposed 1) that the current pulses spread only longitudinally, 2) the intracellular impedance to axial current flow is a simple resistance, and that 3) the membrane acts as a simple condensator leaky capacitor. Although in muscle cells these presumptions can rarely give accurate results for the sake of simplicity, the muscle

fibre was considered to have a cilindrical and not a spindle form. We also assumed that the muscle fibre is of unlimited length. In spite of the objections mentioned above the linear one-dimensional cable theory based on the most simple assumptions seems to be a useful approach.

Results

1. The effect of polarizing current: Fig. 1 shows ETPs obtained in response to the 1 sec hyperpolarizing and depolarizing stimuli. Essentially three types of responses could be differentiated in both cases. The depolarizing current pulse evoked a catelectrotonic potential (see Fig. 1 d and e). On this catelectrotonic response either a single spike or a burst consisting of two or three spikes can be observed. In a few cases such burst responses could also be observed in spontaneously active hearts (Fig. 2). These second type of the responses is presented on the Fig. 1 f, where a small hump could be seen in the raising phase of the electrotonic potential. Action potentials were not observed even if stimuli were increased. The third type of the responses was only a passive reaction of the membrane. Opposite responses were evoked by the hyperpolarizing stimuli. In response to the anodal break an AP or a burst of APs appeared (Fig. 1 a, b). In case of the second type of the responses, the ETP decreased to a steady level after reaching its maximum although the stimulus continued (Fig. 1 c). In the third case, a smooth anelectrotonic potential was observed without AP. If the spontaneously active hearts were stimulated with weak current pulses the spikes did not follow the frequency of these stimuli.

On the other hand after strong anodal current stimulation anelectronic potentials with occasional junctional potentials were generated. (Fig. 3 c, d). This phenomenon, similarly to the groupped discharges of the APs reflected the activity of neighbouring cells.

2. Cable- and electrical properties.

In case of equal stimuli the amplitudes of ETPs of the snail heart muscle cells are in an exponential relation to the distance from the stimulating electrode (Fig. 4). This exponential relationship could be well described by the equation 4. From the plot obtained, the change of the membrane potential (V_o) in the immediate vicinity of the stimulating electrode, the input resistance (R_{inp} = recorded potential/applied current), and the space constant of the muscle membrane could be determined well. The space constant of the membrane is equal to the distance measured at the point where the amplitude of ETP decreased to the $V_o e^{-1}$. In steady state condition, the input resistance of the membrane was 8.3 ± 0.8 Mohm, while the space constant $\lambda = 1.7 \pm 0.1$ mm.

The current-voltage relationship of the muscle membrane was usually S-shaped, but deviation caused probably by different physiological conditions of the muscle were also observed. The current-voltage characteristic of the muscles is presented on Fig. 5. Increasing both the anodal current (open circles) and

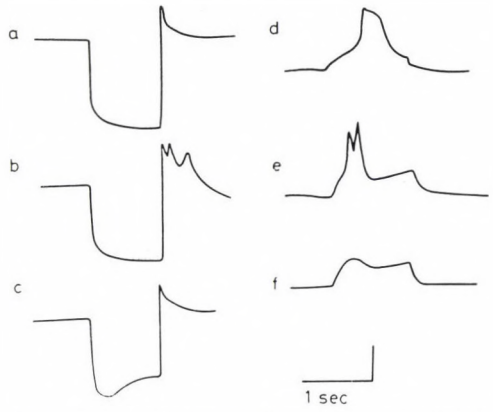

Fig. 1. Active and passive responses of the muscle membrane to the hyperpolarizing (a, b, c) and depolarizing (d, e, f) stimuli. Calibration: 10 mV (a, b, c) and 20 mV (d, e, f)

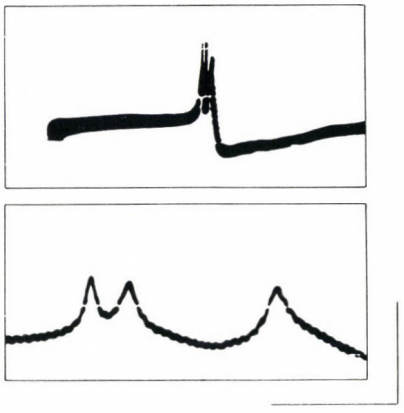

Fig. 2. Groupped discharges of the spontaneously active heart at different time scale. Calibration 20 mV, and 5 and 0.5 msec

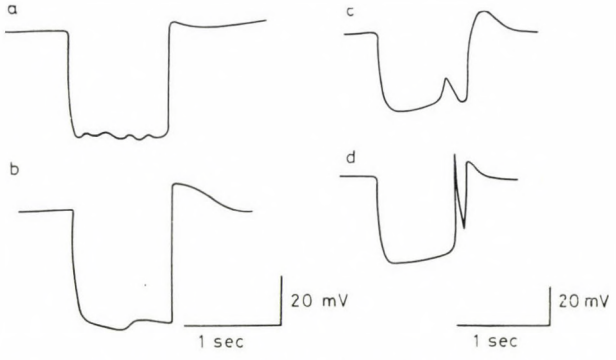

Fig. 3. Junctional potentials on the anelectrotonic responses

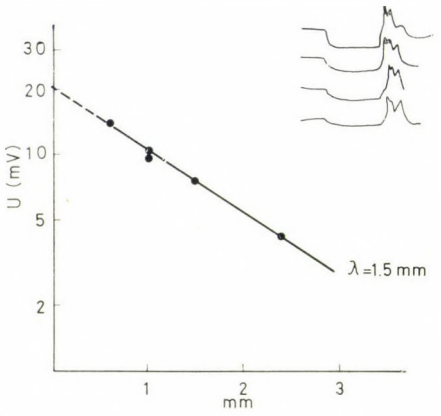

Fig. 4. The relationship between the distance from the source of stimulation and amplitudes of ETPs. Oscillograms show the responses to the equal stimuli with decreasing distance from the point of stimulation

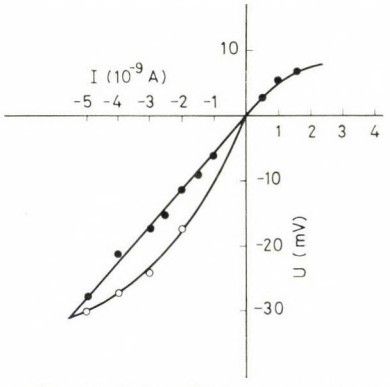

Fig. 5. The current-voltage characteristics of two preparations, which show a rectifier properties in hyperpolarizing (open circles) or in depolarizing (filled circles) directions

the cathodal current (filled circles) a rectifier character of the membrane was found. In hyperpolarizing direction the rectifier properties of the membrane appeared between -20 and -40 mV, and in the depolarizing direction between $+5$ and $+10$ mV. Within these voltage ranges, the current-voltage relationship was almost linear. The cable characteristics of a muscle fibre can completely be defined by the space and time constants of the membrane. The time constant (τ_m) was determined graphically; i.e. the time required to reach 84 per cent of the final value of the ETP. Since erfc(1) = 0.84, V rises to 84 per cent of its steady state value in one time constant, the 84 per cent can give the value of $R_m \cdot C_m$. The mean value of the membrane time constant of snail heart muscle was τ_m 71.4 ± 6.2 msec (Table 1). Considering these conditions the specific membrane resistance and capacitance i.e. $R_m = 20$ kohm $\cdot$ cm^2 and $C_m = 5.4$ μF/cm^2, respectively were determined by Equations 5 and 6 (Table 1).

Table 1

	λ mm	τ_m msec	$R_{\text{Inp.}}$ Mohm	R_m Ohm·cm^2	C_m μF/cm^2
Control	1.7±01. (26)	71.4±6.2 (26)	8.3±0.8 (24)	19 606±3215 (26)	5.4±0.7 (26)
Ca-free	1.27±0.13 (7)	45.0±7.4 (6)	6.6±1.0 (6)	15 654±4876 (8)	4.6±0.6 (7)
$P_{0.05}$	$t > t_{05}$	$t > t_{05}$	$t < t_{05}$	$t < t_{05}$	$t \gg t_{05}$
After glycerol treatment	1.2±0.1 (10)	31.5±7.0 (10)	8.9±2.1 (5)	9 692±2222 (10)	4.5±0.9 (10)
$P_{0.05}$	$t > t_{05}$	$t \gg t_{05}$	$t < t_{05}$	$t > t_{05}$	$t \gg t_{05}$

* S. E. is a standard error of mean. The number of experiments (preparations) is given between parentheses. At $t > t_{05}$ the deviation is significant and $t < t_{05}$ the deviation is not significant.

3. Effect of Ca-free solution on the electrical properties.

The effect of Ca-free solutions on the spontaneous electrical activity and ultrastructure of the heart was described earlier (Kiss, S.—Rózsa, 1971; Elekes et al., 1973). Table 1 contains the changes of the electrical parameters of muscle membrane related to the control. In each case the Student-test was used to define the significance. The change of the parameters means that due to the effect of Ca-free solution the total membrane area decreased and in consequence a part of the cell membranes was disconnected by the Ca-free solution. The cell-to-cell connection is also weakened, which was confirmed by declination of the spatial distances of ETPs- (1.27±0.13 mm). Figure 6 shows amplitudes of the ETP measured in the control and in the Ca-free solution and plotted against the distance from the point of the stimulation. The contractions were blocked in the Ca-free solution. At the same time the AP could be evoked, its parameters however changed significantly: the amplitude and the rate of rise decreased, while the time course of the potential increased. The ETPs showed the decrease of the membrane resistance, however, these alterations were not significant (Table 1).

4. Effect of hypertonic solution:

The electrical parameters of the heart muscle fibres of the snail were investigated after incubation in hypertonic solution. Table 2 shows the values of the MPs obtained during the treatment and incubation again in the control physiological solution. From Table 2 it can be seen that the MPs were practically unchanged in the solution containing 400 mM glycerol even after two hours. The mean value of the MP was −37 mV in the hypertonic and −40 mV in the control solution.

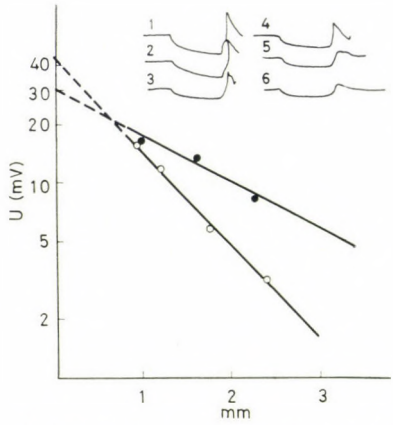

Fig. 6. Effect of Ca-free solution on the spread of ETPs (open circles) comparising to the control (filled circles). Oscillograms 1, 2, 3 represent the control, while 4, 5, 6 represent the effect of Ca-free solution

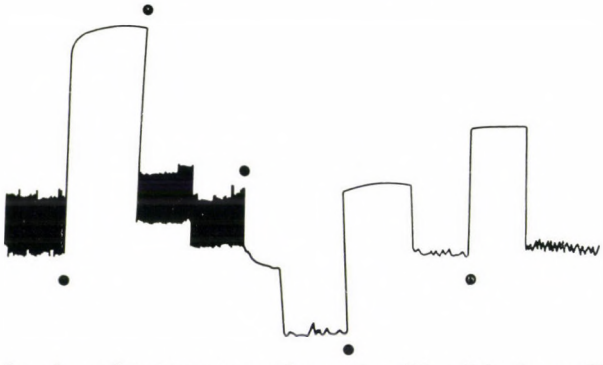

Fig. 7. Effect of the glycerol treatment: on the contractility of the heart. The dark points marked different interactions: 40 mM KCl, physiological solution, 800 mM glycerol, physiological solution, 40 mM KCl

After glycerol treatment, the preparations were replaced into the control physiological solution, and a stable MP could be measured even after three hours (Table 2). This showed that in spite of the osmotic shock the surface membrane maintains its original properties. From 15 preparations 11 showed mechanical activity after glycerol treatment (Fig. 7). The height of contractions, however, decreased to the half value of the control. Even if the contractility was not restored, the potassium-contracture could be elicited (Fig. 7).

The MP was similarly influenced by the hyperpolarizing current before and after the glycerol treatment. The input resistance of the membrane remained substantially unchanged (Table 1). In 50 per cent of the experiments, the AP could be evoked by anodal breake after glycerol treatment (Fig. 8). The time

Table 2

Changes in the membrane potential (mV) in hypertonic solution and after treatment with hypertonic solution

	Control	In glycerol		After glycerol treatment		
		60 min	120 min	60 min	120 min	180 min
1	30.1 (14)	33.7 (4)	30.0 (13)	—	—	—
2	34.2 (6)	29.3 (7)	25.0 ?	24.5 (5)	—	—
3	36.7 (7)	36.6 (6)	26.0 ?	25.9 (12)	—	—
4	51.5 (6)	47.0 (11)	—	57.4 (11)	57.6 (9)	49.8 (18)
5	43.0 (8)	43.8 (6)	44.0 (11)	44.0 (11)	—	46.1 (13)
6	42.5 (15)	45.1 (14)	42.3 (15)	44.0 (12)	40.3 (13)	44.4 (14)
7	43.6 (11)	44.0 (14)	45.1 (12)	45.6 (15)	39.1 (16)	41.2 (17)
8	42.1 (15)	40.3 (19)	44.7 (14)	43.3 (15)	41.9 (16)	43.0 (13)
9	43.3 (6)	—	—	43.5 (5)	—	—
10	43.9 (6)	—	—	40.8 (3)	—	—
11	38.7 (4)	—	—	45.0 (3)	—	—
12	41.2 (5)	—	—	—	—	32.9 (4)
13	40.7 (6)	—	—	—	38.3 (5)	—
14	35.6 (5)	—	—	—	34.1 (3)	—
Mean ±S.D.	40.5±5.0	40.0±5.3	36.7±7.4	41.6±8.8	41.9±7.7	42.9±5.2

constant of the membrane after glycerol treatment decreased essentially (Table 1). The space constant of the muscle fibre also decreased significantly, which means that the effectiveness of the cell-to-cell connection decreased, too.

The specific membrane capacity of the glycerol treated fibers was determined. The capacity decreased by 20 per cent as compared to the control (Table 1).

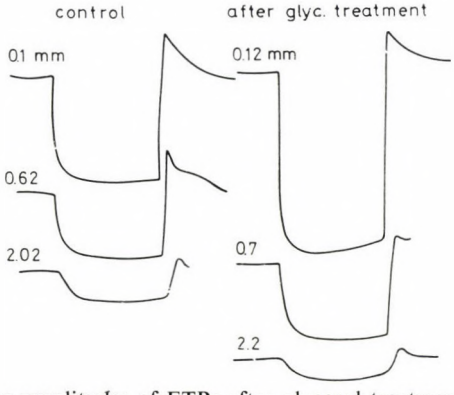

Fig. 8. Changes of the amplitudes of ETPs after glycerol treatment. The numbers show the distance from the stimulating electrode. Calibrations: 20 mV for the first, and 10 mV for the second column

Figure 9 shows the equivalent electrical circuit by which the membrane of the snail heart muscle could be described in first approximation. The parallel linked resistance and capacity (R_m and C_m) represent the surface membrane, while the elements R_1 and C_1 in series represent the sarcolemmal membrane infoldings and the membrane-bound elements of the sarcoplasma, respectively. The rectifier properties of the membrane are not represented on the scheme. It is supposed that these properties originated from the infoldings of the surface membrane and disappeared after treatment with hypertonic solution.

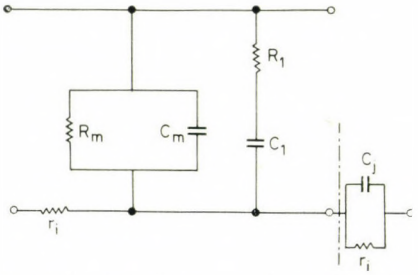

Fig. 9. Equivalent electrical circuit of the heart muscle membrane. R_m and C_m represent the properties of the surface membrane, R_1 and C_1 represent the membrane infoldings and intracellularly located membrane structures

Discussion

1. Cell-to-cell connection

At present, two alternative explanations are known for the cell-to-cell connection in the heart muscle. Tarr and Sperelakis (1964) have suggested that the transmission of excitation between muscle fibres might be chemical in nature. The second, most widely accepted assumption is that the excitation spreads through the low resistance membrane structures by the local circuits (Barr et al., 1965; Weidmann, 1969). This idea was also supported by Procon Yellow staining on frog heart (Pollack, Hustmann, 1973). The muscle fibres of the snail heart possess cable properties, since the ETPs could be registered at more than 2.5 mm distance from the stimulating electrode. The space constant surpassed many times the length of one muscle cell. This is why ETP can be generated only if the cells are interconnected by the low resistance pathways. In snail heart, these structures are presumably the desmosomes, which were found in morphological studies (North, 1963; Elekes et al., 1973).

2. Passive electrical properties of the muscle membrane

On the basis of the cable theory the resistance of the long cilindrical cell is proportional with the diameter of the cell (Hodgkin, Rushton, 1946). The diameter of the snail muscle cells is $7-10\ \mu$ approximately, in compliance with it the input resistance of the membrane is several Mohms. On the basis of the high input resistance, it is expected that the cells can be well stimulated intracellularly. However, at intracellular stimulation AP cannot be evoked. This fact strongly

supports the existence of the functional syncitium in snail heart. On the other hand, the extracellular stimulation proved to be effective, since in this case a number of fibres became excited and the excitation mainly spread along the fibres.

The order of specific membrane resistance is kohm, the voltage-changes of the membrane are slow, according to this the time constant of the membrane is high (75 – 100 msec). The R_m showed a high value as compared with the data obtained on other muscles, but well agreed with the R_m described on the earthworm muscle (Hidaka et al., 1969), on the smooth muscle of Mytilus (Twarog et al., 1973) and on the so called "slow" muscle fibres (Adrian, Peachey, 1965; Stefani, Steinbach, 1969).

It emerges from the current-voltage characteristics that the membrane of the snail heart muscle cell possesses rectifier properties. These rectifier properties could be observed both in hyperpolarizing and depolarizing directions but disappeared after treatment with hypertonic solution.

The capacity of the muscle and nerve membranes differs significantly: in the nerve 1 μF/cm^2, in the muscle 4 – 8 μF/cm^2, in the heart muscle 10 – 12 μF/cm^2, moreover in crayfish muscle 50 μF/cm^2. The higher membrane capacity of the muscle fibres can be attributed to two components: the surface membrane and the transversal tubular (TT) system (Falk, Fatt, 1964). The two components could be separated by the glycerol treatment, and so, the capacity of the surface membrane could be determined. The data obtained on the snail heart have shown that in physiological solution containing glycerol the mechanical activity is restored after a short rest period. This is in agreement with the data obtained on mammalian heart (Strosberg et al., 1972) and on frog ventricular muscle (Fujino et al., 1972). After glycerol treatment, similarly to mammalian hearts, the contractility remained (Niemeyer, Forssman, 1971). The potassium contracture could be elicited but (comparing to the control), the mechanical activity decreased.

On the single basis of the changes observed in the specific membrane capacity, even it is significant, one cannot conclude that the uncoupling of excitation-contraction takes place, because 60 per cent of the contractility of the heart muscle remained unchanged. It may be supposed that the snail heart is not sensitive to glycerol treatment. It was shown by Strosberg et al. (1972) that after glycerol treatment the contractility ceased in the auricular fibres of guinea-pig heart containing no transversal tubular (TT) system. At the same time, contractility was restored after glycerol treatment in the papillary muscle of the same heart which contains TT system. These results do not give an unequivocal explanation of the role of TT system in heart muscle. According to Nelson and Benson (1963), the role of the TT system is rather to insure synchronyzation, than couple excitation-contraction mechanism within one muscle fibre. The heart muscle fibres have a smaller diameter than the skeletal muscles, the time course of APs is also higher, so the depolarization comprehends the whole trabeculae and the Ca necessary for the contraction could be released from the sarcoplasmic reticulum, or Ca ions penetrating into the cell during the stimulate can activate the contractile apparate (Gainer, 1969; Hagiwara et al., 1968).

Our results suggest, that the Ca penetrating into the cell during the AP is the activator of the contraction. The heart, smooth and slow muscles, are characterized by this type of excitation-contraction coupling called "direct coupling" by Bianchi (1969). At the same time, the possibility that the activator Ca is released from the cisternae of the sarcotubular system (SR) located in the vicinity of the surface membrane cannot be ruled out. We have morphological evidence that SR like structures can be found nearest to the inner surface of the membrane, even in close contact with it (North, 1963; Erdélyi, Halász, 1973), and the swelling of these structures was observed after treatment Ca-free solution (Elekes et al., 1973).

References

Adrian, R. H., Freygang, W. H. (1962) J. Physiol. *163* 61
Adrian, R. H., Peachey, L. D. (1965) J. Physiol. *181* 324
Barr, L., Dewey, M. M., Berger, W. (1965) J. gen. Physiol. *48* 797
Bianchi, P. C. (1969) Fed. Proc. *28* 1624
Bonke, F. I. M. (1973) Pflügers Arch. *339* 1
Ebara, A. (1964) Sci- Rep. of the Tokyo Kyoiku Daigaku, Sec. *B12* 1
Ebara, A., Sato, T. (1971) Zool. Magazine (Dobutsugaku Zasshi) *80* 358
Elekes, K., Kiss, T., S.-Rózsa, K. (1973) J. Mol. Cell Cardiol. *5* 133
Erdélyi, L., Halász, N. (1972) Acta Biol. Szeged, *18* 253
Falk, G., Fatt, P. (1964) Proc. Roy. Soc. B. *160* 69
Fujino, M., Yamaguchi, T., Fujino, S. (1972) Jap. J. Physiol. *22* 477
Gage, P. W., Eisenberg, R. S. (1969) J. Gen. Physiol. *53* 265
Gainer, H. (1968) J. Gen. Physiol. *52* 88
Hagiwara, S., Takahashi, K., Junge, D. (1968) J. Gen. Physiol. *51* 157
Hagiwara, S., Henkart, M. P., Kidokoro, Y. (1971) J. Physiol. *219* 233
Hidaka, T., Ito, Y., Kuriyama, H. (1969) J. Exp. Biol. *50* 387
Hodgkin, A., Rushton, W. (1946) Proc. Roy. Soc. *B 133* 444
Howell, J. N., Jenden, D. J. (1967) Fedn. Proc. *26* 553
Irisawa, H., Kobayashi, M., Matsubayashi, T. (1961) Jap. J. Physiol. *11* 385
Irisawa, H., Shigeto, N., Otani, M. (1967) Comp. Biochem. Physiol. *23* 199
Irisawa, H., Irisawa, A., Shigeto, N. (1973) Comp. Biochem. Physiol. *44A* 207
Kiss, T., S.-Rózsa, K. (1971) Acta Physiol. Acad. Sci. Hung. *40* 27
Kiss, T., S.-Rózsa, K. (1973) Comp. Biochem. Physiol. *44A* 173
Kiss, T., S.-Rózsa, K. (1975) Annal. Biol. Tihany, *42* 61
Krolenko, S. A., Adamyan, S. Y. (1967) Tsitologiya, *9* 185
Kuwasawa, K., Hill. R. B. (1972) Experientia, *29* 800
Nelson, D. A., Benson, E. S. (1963) J. Cell Biol. *16* 297
Niemeyer, G., Forssmann, W. G. (1971) J. Cell Biol. *50* 288
North, R. J. (1963) J. Ultrastructure Res. *8* 206
Pollack, G. H., Hunstman, L. L. (1973) Experientia *29* 1051
Sakamoto, Y., Goto, M. (1970) Jap. J. Physiol. *20* 30
Stefani, E., Steinbach, A. (1968) Nature *218* 681
Strosberg, A. M., Katzung, B. G., Lee, J. C. (1972) J. Mol. Cell Cardiol. *4* 39
Tarr, M., Sperelakis, N. (1964) Am. J. Physiol. *207* 691
Twarog, B. M., Dewey, M. M., Hidaka, T. (1973) J. Gen. Physiol. *61* 207
Weidmann, S. (1952) J. Physiol. *118* 348
Weidmann, S. (1969) in Progress in Brain Research *Vol. 31* Akert, K., Waser, P. G. (editors), Amsterdam

Book Reviews

Biologische Oxidation und Citratcyklus by Dargel, R. and Graf, W. (editors) Lehrprogramme der functionellen Biochemie, Teil 2, VEB Gustav Fischer Verlag, Jena, 1975

This book of 130 pages deals in the form of a programmed question-answer system with of the central pathway of the biological transport of material and energy. It constitutes part of a series consisting of five volumes, the other ones being 1. Bioenergetics and kinetics of chemical reactions, enzymology; 3. Carbohydrate metabolism; 4. Metabolism of lipids, proteins and amino acids and 5. Biochemical genetics, protein biosynthesis.

The author's attempt is praiseworthy — when writing this book, he aimed at presenting, his material concerning the cytrate in such a way that it can be used by cycle and the terminal oxidation medical students, students of veterinary college and of biology for self-study. This effort is by all means justified nowadays, considering the problematic state of the informational and educational programs of higher education. These problems are particularly difficult in such dinamically developing fields of science as biochemistry.

Some authors of biochemical textbooks and handbooks published in the past few years attempt to break with writing 'classical' books composed and edited in the traditional way and to offer something new insted, to meet modern requirements. A further aim is not only to systematize the available information in some way or an other but, in addition, to make the reader think to encourage his ability to raise and solve problems or to group the material discussed according to certain practical problems.

R. Dargel's book is an extreme example of such attempts. He aims at leading his reader step by step, in a strict logical order, from the fundamentals of oxidation through energetic problems to the details of the central processes of metabolism. According to the author, comprehension of the material requires only paper, pencil and hard work. Probably for a pedagogical reason, he does not even recommend any handbook in which the reader might find the problems raised in a methodical treatment. At the end of the book he present Pert-program-like sketches by which the connections can be aligned in different orders. The answers are controlled by tests and the reader is informed about his success by a scoring system.

There is surely much pedagogical ingenuity in the very carefully elaborated program system, which allows the student not only to memorize but also to understand the corresponding chapters of energetics and metabolism. In this way it even makes attendance to lectures unnecessary. However, the author fails to disclose how much time a student of average intelligence would need to solve all the exercises and questions properly (neither did the author of this recension have the time to check this). As time is not an indifferent factor in education — being as limited as are many other factors —, it is an essential parameter for evaluating the method offered by the author.

It is doubtless that R. Dargel's educational

program will be very edifying for the instructors of biochemistry and may help them a lot in reconsidering their pedagogical-didactical methods. It is equally doubtless that encouraging unaided thinking is even more important than the proper mastery of the material it is perhaps the most important effort in the instruction of not only biochemistry but of every other subject as well.

P. ELŐDI

H. Reinbothe: *Einführung in die Biochemie für Studierende und praktische Berufe der Biowissenschaften*, VEB Gustav Fischer Verlag, Jena, 1975. pp. 544, 81 figures, 115 tables

For decades the German literature accepted only the term "physiological chemistry" even when the name "biochemistry" had already been widely used in most other languages. This was not purely a terminological problem, but reflected also a certain attitude (ancilla physiologiae). Reinbothe is one of the first authors to have broken with this tradition as reagards title as well as contents and to envisage the compilation of a handbook giving modern biochemical knowledge. In the case of a "hybrid" branch of science that deals with *biological* phenomena by using *chemical* methods, it is very difficult to find the right proportions. The author is inclined to sympathize with the chemical direction and that determines his discussion was done very systematically indeed.

In the introductory chapter, the place of biochemistry among other sciences and its connections with other fields are described. The components of living matter are described in 120 pages (descriptive biochemistry). Before coming to the discussion of metabolism, the role of nitrogen, its cycle inside the living organism and in nature are treated in a separate chapter. This is followed by a review of the basic questions of bioenergetics, i.e. types of energy and the possibilities of their interconversions, with special regard to the role of phosphate in energy transformation. In the chapter dealing with enzymology, mainly of enzyme function and, to a lesser extent, enzyme structure are discussed. The role of subcellular elements in biochemical reactions and transport is then described, briefly characterizing the participation of the individual organelles in various processes. In the next chapter the regulation of metabolism is discussed and this is followed by a separate chapter on biochemical methods, consisting of the description of mutants, innate metabolic diseases and tracer techniques. No doubt these are biochemical methods; however, several others are equally wide-spread in biochemistry. It might have been more appropriate to describe these few techniques and phenomena as methods used particularly in metabolic studies.

The subject of coenzymes is treated in a separate chapter, rather far apart from that on enzymes. The discussion of metabolic processes is divided into individual part each dealing with the metabolism of carbon, oxygen and hydrogen, nitrogen and sulfur, respectively.

It is a common endeavour of the authors of various handbooks to find an appropriate way for the rational discussion of metabolism, a highly complex subjects, and for evading the fact that there are no "handbook" processes and cycles in the cell but there are pools from which the different processes branch according to needs. The way of discussion introduced by Reinbothe is also justifiable, not forgetting, however that there is no such a thing as independent metabolism of carbon, or oxygen, etc.

The subject of the last chapter is protein synthesis and the genetic code. The Appendix contains a list of scientific and educational publications, handbooks and textbooks connected with the individual chapters, most of them written in German and few in English.

The presentation of the book is average; the author of this recension would have welcomed more illustrations as figures are a great help in understanding metabolic processes. The pages appear to be a little crowded. It must be a great help for students that more important notions are set with different letter types (italics, heavy etc.) and some of the equations are enframed, but too much accentuation is rather dis-

turbing. Some biological phenomena, explainable at the molecular level would have deserved more attention, e.g. regulation, excitability, transport, movement etc., all of which are known to appear in some form at all evolutionary levels.

Reinbothe's book most certainly indicates the beginning of a new trend in the compilation of biochemical handbooks (textbooks) written in German. If he succeeds in advancing a little more from the chemical to the biological aspect of biochemistry in a future edition, he certainly will serve more effectively the propagation of those up-to-date theoretical and practical concepts in biochemistry, which we all aim at.

P. ELŐDI

Pathology of Tumours in Laboratory Animals. Volume 1: Tumours of the Rat, Part 1. IARC Scientific Publication No 5, Editor in Chief: V. S. Purusov, Lyon, International Agency for Research on Cancer, 1973, 212 pp.

The International Agency for Research on Cancer devoted a series of volumes to the pathology of tumours in laboratory animals. The aim of these publications is to develop a standardized histological classification and nomenclature of animal tumours. The first volume in this series deals with different tumours of the rat: e.g. those affecting the skin, auditory sebaceous glands, mammary glands, salivary glands, oesophagus, stomach, intestines, pancreas, sofst tissues, bones and the haematopoietic system. Each section contains the description of the normal morphology of the organ concerned, histopathological classification of spontaneous and induces tumours and the principal methods of tumour induction. The large number of illustrations (346) accompanying the papers, will certainly be very useful for the experimental pathologists. The most detailed section of the book is the one dealing with the tumours of the mammary gland. Beside the histopathology of breast tumours, the histochemistry, ultrastructure, transplantation studies, metabolism and growth rate, hormone dependence of induced tumours are included in this section. The book is valuable for the experimental pathologist and other scientists working in cancer research.

O. CSUKA

IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Volume 5: Some Organochlorine Pesticides, Lyon, 1974, 241 pp.

The International Agency for Research on Cancer (IARC) has initiated a program for the evaluation of carcinogenic risk of chemicals to man. Four volumes of this series have been published by the IARC, summarizing the evidence for the carcinogenicity of certain chemicals. The present volume is devoted to twelve organochlorine pesticides e.g. DDT, chlorobenzilate, heptachlor, methoxychlor, the extensive environmental distribution of which is well documented. The sections of this volume consist of four paragraphs.

1. Chemical and physical data: the most important chemical synonyms and trade names are recorded in this paragraph. Chemical and physical properties such as solubility, volatility and stability are also indicated. These properties e.g. lipid solubility might be relevant to carcinogenicity. All data refer to the pure substances. 2. Production, use occurrence and analysis: this paragraph reviews data on the use and occurrence of the chemicals discussed in this volume. The data were mainly collected from the United States and Western Europe. 3. Biological data relevant to the evaluation of carcinogenic risk to man: this paragraph gives information on the metabolic absorption, conversion and excretion of the compounds concerned. Data on carcinogenicity — as determined experimentally in different species — are included together with details on the route of administration and magnitude of effective doses to induce tumours. Clinical and other observations in man, if relevant, are also reviewed. 4. Comments on the data reported and evaluation: this paragraph gives a critical view of the Working Group on the date reported. The book is an excellent encyclopedia for scientists interested in

environmental pollution problems and pharmacologists screening potential carcinogenic agents.

O. CSUKA

Oncogenesis and Herpes viruses. Proceedings of a Symposium held at Christ's Cambridge, England, 20–25 June 1971. Eds: P. M. Biggs, G. de The, L. N. Payne, IARC Scientific Publications No 2, Lyon, 1972 515 pp.

The role of Herpes viruses in the aetiology of animal and human cancers has been discussed in this Symposium. A Herpes virus has been shown to be the cause of a lymphoproliferative and neuropatic disease of domestic fowl (Marek's disease). The Herpes viruses have been closely associated with Burkitt's lymphoma, infectious mononucleosis and nasopharyngeal carcinoma in man and with the Lucke renal carcinoma in frogs. Separate chapters deal with the pathology (1) virology and immunology (2) and epidemiology (3) of the above mentioned Herpes virus-induced diseases.

Virology is the most detailed part of the three chapters dealing with Marek's disease (DM). Serological techniques used for the detection of in vivo and in vitro induced antigens, comparative studies of the DNAs of MD viruses and are included in this section. Since the Lucke renal carcinoma of frogs is of little practical importance, only a short section is devoted to this pathological alteration. The presence and aetiological role of the Epstein-Barr virus (EBV) in Burkitt's lymphoma and infectious mononucleosis are supported by several studies of this Symposium. It is claimed that nasopharyngeal carcinoma is associated with an infection by a Herpes-type virus immunologically very similar to the EBV. The role of Herpes simplex virus (type 2) in the induction of cervical cancer is also considered by several contributors. The book promotes our understanding of the role of viruses in the induction of cancer.

O. CSUKA

Mitochondria. Bioenergetics, Biogenesis and Membrane Structure. Edited by L. Packer and A. Gomez-Buyou. — 406 pages. Academic Press, Inc. New York–San Francisco–London 1976

This volume contains the lectures presented at an advanced course held in June 1975 in Mexico, on the biogenesis, structure and function of mitochondria. Most individual articles are therefore necessarily of a review character and offer a comprehensive survey of the field. It is the merit of the organizers and the sponsors of the symposium that these reviews were given by the best authorities, who contributed significantly to the success of the course. The texts presented are both basic and advanced and include all essential elementary informations as well as some of the most recent, partly unpublished results.

According to the reviewer it is the section on biogenesis which contains the most complete summary of its topic. Five reviews offer a comprehensive survey and these are followed by a research paper. Linnane et al. give both the theoretical background and the experimental approach as well as the possible use of continuous culture of yeast cells. Mahler reviews the biosynthesis and structure of proteins which are composed of several subunits, i.e. cytochrome oxidase and the oligomycin sensitive ATPase as well as the integration of the subunits into a single functioning enzyme; a new class of mitochondrial mutants was used in this study. Tzagoloff summarizes cytoplasmic and nuclear mutations that affect mitochondrial function. Beattie wrote a chapter on mitochondrial membrane formation and finally Griffiths described the different mutants which are of use in the study of oxidative phosphorylation with a special emphasis on drug-sensitive mutants.

The papers on bioenergetics cover the most important current trends. Cation transport is the subject of 3 while anion transport of 4 chapters. Two different aspects of calcium transport are covered by Scarpa and by Garafoli, the latter presenting the present status of our knowledge about the mitochondrial calcium-binding protein which was postulated to be the calcium-carrier. Brierley deals with monovalent cation trans-

port. LaNoue and Tischler treat metabolite transport at the mitochondrial membrane level while Williamson reviews the role of the mitochondrial metabolite carriers in the economy of the cells. The adenine nucleotide carrier is the subject of two chapters, written by P. M. Vignais et al. and by Klingenberg and these give a full account on the status of adenine nucleotide transport. The important and recent trend of reconstituting various mitochondrial functions by the incorporation of purified proteins into liposomes is covered by Hinkle on the basis of the chemiosmotic hypothesis. The same hypothesis but at the yeast cell membrane level forms the basis of the research paper of Pena dealing with proton transport. 3 further research papers, one on mitochondrial enzyme localization (Rendón and Packer), one on the action of guanidines (Tuena de Gomez-Puyou et al.) and finally one on electron transport (Estrada-O and Gomez-Lojero) complete the section on bioenergetics.

The third section is devoted to membrane structure and is somewhat more heterogenous than the previous parts. The papers in this section are also less of a review character and mostly contain research reports. They are, however, very useful as recent physical techniques introduced into membrane research are described including spin labelling (Keith, P. M. Vignais), NMR studies (Cerbón) fluorescence, both conventionel (Klip and Gitler) and nanosecond fluorescence spectroscopy (Fortes). Chemical modifications of membranes, including surface probes and bifunctional reagents were presented by Tinberg and Packer. Darszon and Montal reported on rhodopsin — phospholipid bilayer vesicles.

The readability of the text is excellent, the quality of illustrations is very good. The volume is strongly recommended for both the beginners and also for those who want to find the most recent information on mitochondrial function.

A. FONYÓ

The Structural Basis of Membrane Function. Proceedings of the International Symposium, May 5–7, Tehran. — Edited by Youssef Hatefi and Lisa Djavadi-Ohaniance. 479 pages. Academic Press, Inc. New York–San Francisco–London, 1976

It is generally agreed by now that research activity is essential not only in the developed but also in the developing countries. The Symposium organized in 1975 in Iran by the Department of Cellular and Molecular Biology of the University of Tehran and sponsored by the same University, served this purpose and achieved a high-level scientific meeting. It also established contacts between Iranian scientists and scientists coming from the most advanced laboratories. The lectures delivered at the Symposium represent well selected summaries of the present status of current problems as well as of major trends in membrane research.

The topics were arranged into six sections and as one of the organizers pointed out in his foreword, these six aspects resemble the six faces of the same cube. The sections were: membrane structure, transport, energy-transduction, regulatory function of membranes, excitable membranes and finally microsomal and related membranes. The volume is headed by the IUB lecture given by Philip Handler, President of the National Academy of Sciences of the U.S. on "Science and the Developing Nations". The last chapter in an extremely clear Summary of the Symposium by F. M. Huennekens.

It is a necessary consequence of the organizational practice of symposia that related topics are scattered through different sections and therefore the titles of the sections do not reveal the actual content. This happened also to mitochondrial bioenergetics and biogenesis. The volume contains the most up to date concepts, data as well as speculations on bioenergetics. We find a summary of the brilliant research on structure and biosynthesis of the cytochromes in yeast by Schatz; recent results concerning the mitochondrial adenine nucleotide carrier by the Klingenberg group: biochemical genetics of the oxidative phosphorylation system by Griffiths: the structure of the mitochondrial ATPase as revealed by phys-

ical methods by Penefsky and his group; new data on the quantitative relationship between proton extrusion and calcium uptake highly revelant to the chemi-smotic theory of oxydative phosphorylation by Lehninger and Brand. The present status of the "conformational coupling theory" of oxidative phosphorylation can be evaluated by the papers of Slater, and those of the Boyer group. Electron transport is summarized by Hatefi et al., while charge separation which occurs during electron transport is treated by the group of Papa. New aspects of ion transport in mitochondria are presented by Azzone et al.

While 12 papers out of 30 deal with mitochondria, other fields of membrane biology are also well covered. Metcalfe et al. report on a phospholipid annulus which surrounds the ATPase of sarcoplasmic reticulum involved in calcium transport. Stoeckenius summarizes how the ion pump containing bacteriorhodopsin is organized. Transport in bacterial membrane vesicles and its molecular mechanisms are reviewed by Kaback et al.

A lucid survey of the molecular physiology of muscle is given by Huxley: it deals with the signal for contraction, the nature of the contractile process and the mechanism of regulation by calcium and it also includes recent data on muscle proteins. Another excellent chapter in molecular physiology is that on excitable membranes: the sodium channels are reviewed by Keynes and the synaptic receptor proteins by DeRobertis.

The volume also includes papers on the mechanism of action of certain highly active compounds at the membrane level: aldosterone (Rasmussen et al.), cholera toxin (Cuetrecasas et al.), gramicidin a (Bamberg et al.), Microsomal electron transport (Estabrook et al.) as well as the cytochrome P-450 hydroxylation system (Coon et al.) are included. The possible role of the P-450 system in the metabolism of polycyclic hydrocarbons and their relation to chemical carcinogenesis are treated by Ernster et al. An interesting aspect of membrane biology is the role of defects of amino acid transport in diseases, summarized by Meister.

The volume is produced by the AP rapid manuscript reproduction technique. In contrast to the variable quality of similar volumes, due to poor manuscripts, this one represents superb readability because the entire text was retyped by the Publisher.

A. FONYÓ

Neuronal Recognition. Edited by Samuel H. Barondes is the second volume of a series in Current Topics in Neurobiology, Plenum Press N. Y. London, 1976

The book consists of three main parts. The first deals with specificity in synaptic development and regeneration, the second with morphological and biochemical studies of synapses and the third with the molecular basis of neuronal recognition.

In the first section the degree of specificity in the retinotectal and the nerve-muscle system is analyzed. The effects of deafferentation on new synapse formation in the embryo and the expression of neuronal specificity in tissue culture are also stressed.

The second section contains reviews of morphological studies of synaptogenesis and biochemical studies of synaptic components. It deals with the isolation and characterization of the chemical components of the synaptic function.

In the third section specific molecules are described that might mediate cellular recognition. A major question is whether molecules of this type even exist. The role of membranes as a tool for studying cell surface recognition is emphasized and the way of selecting the best material for this type of investigation is described. Experiments on slime molds are also mentioned in this relation.

The authors of the book provide us in most of the difficult questions with tentative answers only, but the problems seem to be neither premature nor hopeless.

In conclusion, Neuronal Recognition, deals with a difficult but solvable topic and offers several interesting results from this field.

MÁRIA WOLLEMANN

Protein Crystallography by T. L. Blundell and L. N. Jonhson. Molecular Biology, An International Series of Monographs and Textbooks, Academic Press London, New York, San Francisco, 1976. 565 pages

This book represents an introduction to protein crystallography on one hand and a review of the achievements in this field, on the other. The authors attention is focused on the application of the techniques of X-ray diffraction. In addition, however, neutron and electron diffractions are also dealt with. At present a tremendous amount of materials is scattered in various journals and reference books. In this book a fortunate selection of theoretical, technical and experimental aspects of this topic is presented. The authors effort to present a comprehensive review especially on "protein" crystallography is completely successful and the differences between the protein crystals and crystals of small molecules are emphasized. Since the book is directed to both crystallographers interested in protein research and biochemists concerned with molecular structure, the introductory part includes a brief explanation of the principles of protein structure and those of X-ray diffraction.

The authors give a short historical summary of this young discipline, describe the steps in the X-ray analysis of protein crystals and discuss all the various aspects of the subject in a comprehensive and detailed fashion. They described the crystallization of proteins, the symmetry and optical properties of protein crystals, isomorphous replacement, anomalous scattering, preparation of heavy atom derivatives, data collection, data processing, the determination of heavy atom positions, the calculation of phases, the interpretation of the electron density map, difference fouriers, refinement procedures etc. A more detailed and critical description of the instrumental techniques would help research workers to select the proper equipment. Unfortunately, however, only very little space is devoted to these problems.

The book concentrates on those methods which proved successful in structure determinations. The selection of the results to represent the achievements of protein crystallography is representative rather than comprehensive or arbitrary. Here we find the stereo specificity of lysozyme, the sequence of steps in the reaction of haemoglobin with oxygen, and the structure of F_{ab}, a fragment of immunoglobulin.

The book is a useful reference work for research workers in biophysics and biochemistry, for people teaching in the field of molecular biology or crystallography and for postgraduates commencing research.

There are more than 500 well-selected references, a useful bibliography of the papers scattered in a wide variety of journals in physics, chemistry, biochemistry and crystallography. The format is good, the typeface is easy to read and it is well illustrated with figures, schemes and tables. The book is well written and has cohesion.

P. ZÁVODSZKY

Methods in Membrane Biology Vol. 7. Edited by E. D. Korn Plenum Press — New York and London 1976 XV + 267 pages

The extensive and complex interdisciplinary research in the field of membrane biology raised a lot of question about membrane organization and the topological distribution of membrane lipids and membrane proteins. The application of new techniques providing complementary information about the structure and function of membranes demonstrated that most of the membrane lipids are definitely in a bilayer array. However, the bilayer and some specific phospholipids, as well as an asymmetric distribution of phospholipids within membranes were also shown to exist. The new physical a lot about membrane architecture. This volume is devoted to these new techniques and it also introduces unpublished data about the ultrastructure of membranes. In the first chapter electronmicroscopic methods in membrane biology are discussed and a description of the principles and technique of electron microscopy as well as various methods of sample preparation are introduced. Special attention is paid to the freeze-etching technique, the technique which gave evidence that proteins might penetrate

through the membrane phospholipid bilayer. The authors — H. P. Zingsheim and H. Plattner — have made a successful attempt to describe the kind of information one can derive from electron microscopy.

There is a full discussion of supplementary methods such as autoradiography, morphometry, specific staining reactions, cytochemistry, immunochemistry, localization of ions, etc. A critical analysis of the potentials and limitations of the methods is also given. The chapter concludes with a brief review of the results achieved by using electron microscopic and complementary techniques in membrane biology. The format is good, the chapter is illustrated by 18 good quality photographs and figures, there is a useful bibliography of more than 600 references.

In the 2nd chapter B. Roelofsen and R. F. A. Zwaal illustrate the usefulness of an enzymatic approach to the determination of membrane phospholipid distribution by a detailed description of the application of this method to the human erythrocyte. The preparation of highly purified phospholipase A_2, phospholipase C and sphyngomielinase is described. The asymmetric distribution of major phospholipids on the two sides of the erythrocyte membrane bilayer is an interesting unanticipated finding. There are 48 references concerning this, special field of membrane research.

The next chapter is devoted to methods of labelling cell surface carbohydrates. The authors, C. G. Gahmberg, K. Itaya and S. I. Hakamori, provide all the necessary experimental details for the chemical and enzymatic labelling of the surface carbohydrates and for the separation and identification of the labbeled molecules. There is also a good summary of the literature in the field.

It was a good choice to include chapter 4 about phospholipid exchange between membranes. In the first three chapters membrane organization and the topological distribution of membrane lipids are discussed and the asymmetrical distribution of phospholipids in some membranes is demonstrated. The last chapter deals with the way this specific distribution comes about. The contribution of D. B. Zilversmit and M. E. Huges concentrates primarily on the purification of phospholipid exchange proteins and the methods of assaying phospholipid exchange activity. The use of phospholipid exchange protein to study the properties of cell membranes is also discussed.

P. ZÁVODSZKY

Function and Metabolism of Phospholipids in the Central and Peripheral Nervous Systems by Porcelatti, G., Mamaducci, L. and Galli, C. Advances in Experimental Medicine and Biology, Vol. 72, pp. 412. Plenum Press, New York and London, 1976

The volumen comprises all the papers delivered at the International Satellite Meeting (Cortona, Italy, August, 1975) of the 5th International Congress of the International Society for Neurochemistry (Barcelona, 2—7 September, 1975). The papers are centered around the following topics: Metabolism and Turnover of Phospholipids in Nervous Tissues (11 papers),

Aspect of Phospholipid Function in the Nervous Systems (7 papers),

Phospholipids in Brain Damage (5 papers),

Pharmacological Action of Phospholipides on the Nervous System (4 papers),

Structural Requirements for Phospholipids in Biological Membranes (3 papers).

Experts in the field of biochemistry, biophysics, physiology, pharmacology and pathology discuss various aspects of the metabolism of phospholipids and plasmalogens in nervous systems and also in some other tissues. The papers presented contain up about the biosynthesis of these compounds in nervous tissues as well as about the metabolic effects of intact phospholipids. Effects of neurohormones and their antagonists on the metabolism of some phospholipids (e.g. phosphatidyl inositol) are discussed in detail. Several papers (those dealing with turnover of brain phosphoglycerides or with the various routes of phospholipid biosynthesis) outgrow the limits of lipid neuro-chemistry and contribute a great deal to our better understanding of the biochemistry of these substances in general. Other papers give useful techniques for isolating brain subcellular fractions (myelines

and nerve endings) or intermediers of phospholipid metabolism (such as CDP-diglycerides). The volume is of great value for scientists working in the field of lipid biochemistry and physiology. In combination with books furnishing basic information about the analysis, composition and metabolism of phospholipids it is well suited also for teaching this subject to advanced students.

T. FARKAS

Jiri Sevcik: *Detectors in Gas Chromatography*. Journal of Chromatography Library Volume 4, Elsevier Scientific Publishing Company Amsterdam—Oxford—New York, 1976 (192 pp)

Very few books have been published so far on detectors used in gas chromatography, although the availability of sensitive, reliable detectors — including general purpose and so-called specific detectors — contributed significantly to the widespread application of gas chromatography. The book of Jiri Sevcik now fills this gap. The author deals with almost all kinds of gas chromatography detectors: the thermal conductivity detector, the electron capture detector, the flame ionization detector, the thermoionic detector using an alkali metal salt, the photoionization detector, the helium detector, the flame photometric detector, the coulometric detector, the electrolytic conductance detector.

The main value of the book is that it evaluates the main features of the various detectors: detector signal (linearity, sensitivity, selectivity), effect of experimental conditions on the detector signals application.

Although it is not the intention of the author to cover the whole literature, at the end of each chapter we find a list of references on detection mechanism and experimental conditions.

The book is very helpful for those who want to purchase gas chromatographs and also for those who wish to extend the efficiency of their gas chromatographs by fitting in new detectors.

A. VÁRADI

Proteins and Related Subjects Volume 23 *Protides of Biological Fluids* (Ed.: H. Peeters) Pergamon Press, Oxford—New-York—Toronto—Sydney—Paris—Frankfurt, 1976

This 699 page volume comprises 117 papers, the proceedings of the 23rd Colloquium on "Protides of Biological Fluids" held at Bruges in 1975. Three main topics were discussed: Proteinase Inhibitors, Clinical Evaluation of Plasma Protein Patterns, and Affinity Chromatography. Some of the most interesting results published in this book will be presented below.

Proteinase Inhibitors are discussed in the following sections (46 papers): Proteinase Inhibitor Interactions, Structure and Function of Human and Animal Proteinase Inhibitors.

14 papers deal with the interactions of inhibitors e.g. human-1-proteinase inhibitor, a_1-antitrypsin and a_2-macro-globulin. In one paper the interaction of different macro- and low molecular weight inhibitors with blastocyst proteinases involved in implantation is presented. It could be concluded from this study that rabbit blastocyst enzyme might be related to elastase rather than to trypsin-like or chymotrypsin-like proteinases. More information is given about the structure of protein-proteinase inhibitors mentioned above, the thrombin inhibitor hirudin, and also about that of a low molecular weight elastase inhibitor from human serum and the isoinhibitors isolated from human seminal plasma. One of the latter, HUSI-II, seems to be the inhibitor of acrosin (the acrosomal proteinase important in the fertilization process) while the other, HUSI-I, has been assumed to belong to the special trypsin-chymotrypsin inhibitors like those isolated from the human respiratory tract and cervical mucus. The possible role of proteinase inhibitors in cell growth and in cartilage metabolism is also discussed. Isolation and structural studies of some new or quite recently discovered inhibitors from animal tissues are also described, information is presented, for instance, about the Japanese quail ovomucoid inhibitor and about the inhibitors isolated from dog submandibular glands and from See Anemones, as well as about

snail sectory inhibitors. The latter exhibits strong inhibitory action against enzymes produced by soil bacteria. In this work a modification of the automatic sequencing method is mentioned, which allowed unequivocal assignment of all amides (Asn, Gln, terminal $CONH_9$).

Concerning the studies on proteinase inhibitors one of the authors' opinion is worth quoting: "The wide distribution of proteinase inhibitors in nature as well as the conservation of key structural elements during evolution indicates that these substances probably play a more general, and fundamental, role in biological processes than had been anticipated solely on the basis of our knowledge of pancreatic proteinase inhibitors".

In the first subsection, among others, an automated approach to the preparation (or rather determination) of nine plasma proteins and the use of laser nephelometry are discussed. Six papers deal with evaluation methods, i.e. the trace plasma proteins and their possible value for clinical diagnosis. Here we can also read about the isolation of nine trace plasma proteins (including a_1-antichymotrypsin) by means of the combines Rivanol ammonium sulphate procedure. Two papers discuss the plasma protein profiles in pregnancy. The subsection of Profiles Typical of a Disorder contains a detailed description of the α-antitrypsin system and includes a new procedure for the isolation of α-antitrypsin by using thiol-disulphide interchange which provides large amounts of the substance with full immunological and proteinase-inhibiting activity. Five papers are devoted to immune disorders and another five to liver and renal decreases. One of them shows a possibility to prognosticate rejection crisis at kidney transplantation. Seven papers of the last two subsection give information, for instance, about the plasma protein level in arthritis and the role of known hormones (e.g. corticosteron) in the regulation of net biosynthesis of five acute phase plasma proteins (albumin, fibrinogen, -acid glycoprotein, a_2-globulin, and haptoglobin).

A comprehensive review is presented about the crosslinking of agarose (Sepharose) by different agents (e.g. 2,3-dibromopropan-1-ol, divinylsulphon), about the properties of crosslinked matrixes as well as about the coupling of ligands to solid supports. In addition to the well-known CNBr activation the use of "photo-beads" and of SH-containing gels (e.g. Sepharose with glutathione moieties) is also outlined. Peptides and proteins can be attached to photo-beads by photoalkylation. In this reaction the CH_2 groups of Gly residues present in the protein are alkylated by the methyl group of toluene residues or olefinic double bonds of alkyl side chains of the matrix. Irradiations are carried out in the presence of a sensitizer (e.g. 2-acetyl-benzoate) in aqueous solutions. Enzymes fixed on resins this way, i.e. through the peptide backbone instead of a side chain function, possesses high activity. SH-Sephadex can be used for affinity chromatography as well as for covalent chromatography. A very successful purification of papain is described to exemplify the latter method. Antigen-antibody purifications, isolation of enzymes and viruses are described in 22 papers. For hydrophobic interaction chromatography alkyl or amino-alkyl moieties are attached to Sepharose through ether or carbamic ester bonds. Pentyl side chain proved to form stronger hydrophobic interactions than the phenyl or benzyl residues and the ether linkage seems to be more advantageous than the carbamic ester bond. The latter exhibits electrostatic interactions too and, due to the two types of adsorption, chromatography on such gels frequently gives irreproducible results. Isolation of plasma proteins and that of lipoproteins are shown as examples of hydrophobic interaction chromatography.

S. BAJUSZ

Thermodinamics of the Polymerization of Proteins by Fumio Oosawa, Sho Asakura In Molecular Biology, an International Series of Monographs and Textbooks. Editors: B. Horecker, N. O. Kaplan, J. Marmur and H. A. Sheraga. Academic Press, London, 1975

In this work the process of the organization of protein molecules into subcellular units is discussed from a thermodynamical aspect. The authors summarize our knowledge about the thermodynamics of protein polymerization partly on the basis of their own experimental and theoretical work and partly from the literature. They concentrate mainly on those problems of protein polymerization which seem to be common with different protein polymers. Though experimental work has been carried out on in vitro systems, tentative conclusions for in vivo systems are also drawn where this is possible. The chapters, following each other in a logical order, deal with the theory of polymerization equilibria, the kinetics, energetics and regulation of polyperization, the structure and motility of polymers and with highly ordered structures.

The wide use of this book is made possible by the fact that the mathematical and physical skill required does not exceed the usual subject-matter of universities even when discussing very complicated theoretical problems. Comprehension is greatly aided by 79 very informative figures and further studies or a deeper understanding of the individual problems by 245 references.

The book is recommended to biophysicists, biochemists, to physicians doing research in the field of molecular biology, to crystallographer and other reserchers of ultrastructure.

I. SIMON

Subnuclear Components: Preparation and Fractionation. Editor: G. D. Birnie. Butterwoth Publ. Co., London, Boston, 1976. pp. 334

After the success of "Subcellular Components: Preparation and Fractionation", G. D. Birnie has edited a splendid method logical handbook about the components of the cell nucleus.

The book is significantly more than a simple compilation of some of the most useful methods. First of all, the purity and composition of the subnuclear preparations obtained by the individual methods are clearly denote. Attention is drawn to some considerations which will help to chose proper isolating medium and the physical and chemical conditions (ionic strength, pH, centrifugal force etc.). It is stated to what extent these conditions may be varied and what the consequences of their variation are. The authors offer their motives for reviewing just the methods listed and they often point to the disadvantages of those procedures which are not described in detail. They compare different methods and discuss their advantages, limits and disadvantages. It is especially valuable that the procedures of isolation of subnuclear components are grouped and — in most cases — arranged in a tabular form according to cell sources, the tables containing all the important data, from isolation media to references.

Parameters are given by which the biochemical and biological purity (and activity) of each subnuclear component can be tested.

Some of the methods, considered particularly useful by the authors, are described in sufficient detail to be casi by reproducible in the laboratory. When reading these descriptions one has the impression that the authors are not only excellent specialists in the field of subnuclear components and not only known the preferred method(s) but have also worked with most of them. Thus, their critical approach and advice have a sound foundation.

Editor G. D. Birnie composed a highly valuable book: First of all, he entrusted the best known experts with the compilation of the chapters on the individual subnuclear components. He could also achieve that all the authors accepted the above points of view.

Thus the book is written according to a uniform scheme: it presents well established and profitable in a critical aspect.

In Chapter 1, written by A. Smuckler, M. Koplitz and E. D. Smuckler, the isolation of nuclei is discussed. As the success of the isolation of all subnuclear components

depends upon the quality of the starting nuclear preparation, this chapter constitutes the basis of all the other ones describing the isolation of subnuclear particles.

The subject of the following chapter, written by D. J. Fry is the isolation of the nuclear envelope.

A great merit of both chapters is that qarallel electronmicroscopic observations carried out at every stage of the purification procedure are considered to be one of the most important criteria of purity. It is also pointed out that not only representative pictures are required. However, this requirement is not stressed enough by the authors of the remaining six chapters.

U. E. Loening and Anna M. Baker describe the methods of the isolation of the nucleolus. Owing to the specific role of this particle in ribosomal RNA synthesis, the authors had a good opportunity to point out many functional elements and criteria.

Naturally enough, the chapter written by D. Rickwood and G. D. Birnie on the complicated and exciting topic of chromatin completely fulfills the general concept of the editor. The sections dealing with the template activity and fraction of chromatin are particularly valuable. The description of the fractionation and isolation of histones may have been a relatively simple task for E. W. Johns, the problem itself being more settled. This is followed by an exciting chapter about the non-histone proteins of chromatin by A. J. MacGillivray, which includes a detailed description of the most up-to-date two-dimensional separation techniques.

The book ends with chapters on nucleic acids. In this chapter about the isolation of nuclear RNAs, M. E. Bramwell circumstantially discusses RNA degradation and aggregation in the course of isolation. However, this field is so much in the focus of interest now — because of different RNAs located in the subnuclear particles (pre-messenger RNA or HnRNA, ribosomal RNA and low molecular weight RNAs) — that perhaps this chapter may have deserved a discussion in more depth, especially with regard to the RNAs of the individual subnuclear particles. The last chapter in this book is DNA isolation written by H. W. Butterworth. Here, particular emphasis is laid on the most recently described procedures allowing the isolation of DNA chains of the greatest attainable length.

This book is an excellent introduction and a reliable manual for those who wish to isolate subnuclear particles for the first time. For those who already have working experience in this field, on the other hand, it represents a useful and highly valuable handbook and, owing to its uniform principles of composition and critical aspect, an enjoyable and inspiring reading.

E. J. HIDVÉGI

Mammalian Cell Membranes edited by G. A. Jamieson and D. M. Robinson, Butterworths 1976. Vol. 1. 276 pages.

As a result of experiment and discoveries revealing a highly dynamic structure of cellular membranes a new era of "membranology" has begun to develop in recent years. The quick reactability and adaptability of the plasma membrane towards the environment despite the strict maintenance of the "milieu interior" by the interacellular membrane system has become, once again, a great challenge in many fields in biology. Therefore, it is very timely to issue detailed reviews of a multidisciplinary nature as a guidance. Such reviews should help to develop a common language among scientists of various disciplines who work on the same subject i.e. cellular membranes. This objective is fully accepted by the authors of the ten chapters in the first volume of a series devoted to the "General Concepts". The contributors — G. G. Rose, D. M. Robinson, Mary C. Glick, Y. K. Levin, D. Chapman, P. B. Canham, R. H. Hinton, E. Reid, D. Gingell, S. K. Malhotra, J. Lenard and R. Landsberger — cover very broad areas on the anatomy, separation and cultivation of mammalian cells, the isolation, physical, physical chemical and enzymological characterization of the cellular membranes as well as their functional and biogenetic aspects. The list concludes

with a review on the models of membrane structure.

The volume seems to be fairly complete in itself, and the not yet published coming volumes, — "The Diversity of Membranes", "Surface Membranes of Specific Cell Types", "Membranes and Cellular Functions", "Responses to External Influences" — promise an almost full coverage of up-to-date theories and methods.

Of course, advances in our present understanding of the cell membrane system have been made by using various approaches such as electron microscopy techniques, physical and chemical techniques on model and natural systems, biochemical separation and subsequent analysis — and these are well reflected through the various chapters. Unfortunately, description of autoradiography techniques and of results obtained by them is completely omitted although autoradiography has helped to reveal important metabolic processes concerning e.g. their localization within the various compartments of the cell. One can only hope that such a chapter in one of the forthcoming volumes will help complete this good review.

Some of the principles and results of lectin-binding experiments were collected, but no detailed description of the possible extension of receptor mapping is included, although the application of homologous or heterologous biological materials binding specifically to certain receptors or groups is becoming a powerful tool for studies on arrangements of various membrane components.

In a very rapidly growing field it is rather difficult to provide a critical evalution of all that has been done so far and therefore it is hardly justified to try to restrict the directions of further research or application of methods as proposed in a few chapters of this volume just for the sake of a better comparability of the results through the uniformity of methods. The standardization of only the well-advanced techniques is justified. The questions and problems, however, put forward are mostly stimulating and they might really help to avoid much duplication of work in this vast field.

The reader gets a good orientation in the subject and further guidance is given through the references most of which has come out only in the last 4—5 years.

Despite a few shortages mentioned above the first volume provides a substantial information source for those who are already working in this field and for those who prefer to use these basic results for practical purposes in the applied sciences.

G. J. KÖTELES

Physiology of Smooth Muscle Edited by Bulbring, E. and Shuba, M. F., North Holland Publishing Co., Amsterdam, 1976. 438 pages, ISBN O-89004-051-6

This volume contains the proceedings of a symposium on smooth muscle held in Kiev in September, 1974, as a satellite meeting of the 26th International Congress of Physiological Sciences. The aim of the symposium was to bring together researchers from various fields of physiology, morphology and biochemistry, to analyze the mechanism controlling the spontaneous as well as the drug induced activity of smooth muscles of different origins.

The symposium was divided into five sections, accordingly, the volume consists of five distinct parts:

1. Ionic Composition and Transmembrane Ion Currents in Smooth Muscle. This part presents observations, and speculations on as well as mathematical analyses of the cellular mechanisms controlling ion distribution and ion movements in gastrointestinal, vascular, ureteral and uterine smooth muscles.

2. Origin and Characteristics of Spontaneous Activity in Smooth Muscle. The authors provide information on the mechanical and electrophysiological characteristics of smooth muscles located in different organs.

3. In Part 3 the nature of the electromechanical coupling and the mechanisms of muscles activation are discussed. The studies in this part of the volume are based on some fundamental findings described in Sections 1 and 2.

4. Part 4 describes the fine structure of smooth muscle cells and the attached nerve terminals. The morphological and biochemical findings are discussed in the light of a functional connection between the structural elements.

5. In the last part studies on the mechanism of action of transmitters, modulators and other drugs on the smooth muscle, are presented. The possible physiological significance of the interactions of different drugs with respect to the functioning of the smooth muscle, is also discussed.

A. RÓNAI

R. Glaser: *Einführung in die Biophysik*, 2. Auflage. Gustav Fischer Verlag, Jena, 1976, 349 pages DM 36,50

"Introduction to Biophysics" is the title of this interesting book of about 300 pages. In chapter 2 it discusses "The Molecular Structure of Biological Systems" on ~ 100 pages, in chapter 3 "The Thermodynamics of Biological Systems" on ~ 100 pages, in chapter 4 "Kinetics of Biological Systems" and in the last one "The Biological System in Correlation with its Inanimate Environment" on 50 pages each. — The subject index does not mention stimulus and excitation but contains 15 references to "Elektrodes"; "Membrane" is dealt with from 32 aspects, "Muscle" is described as follows: (p. 119) fluidity of muscle membrane, (p. 147) work of contraction (as a specific case of the Gibbs equation, five lines), (p. 162) (e.g. muscle contraction) (that's all), (p. 265) e.g. "electric excitation of muscle" (in connection with control processes), (p. 279) heat conductivity of muscle (among other materials), (p. 295) muscle power at flight of a colibri. Ununderstandable disaccord in selecting the questions being dealt with in this book appears e.g. in the fact that the process of hearing is described in pages 297—302, whereas optics is represented by the single expression (p. 52) "Sehvorgang" (seeing process).

The content of this book can possibly be of interest for experts of biophysics, the reviewer cannot help raising the question whether an "*introduction*" to biophysics would not be more advantageous for younger colleagues if it described fewer valuable consideration and more biofunctions according to mentality of Du Bois-Reymond, Fick, Ludwig, Müller similarly to Granefield's lecture at The First Proceedings of the USA Biophysical Society (1959)?

E. ERNST

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Mechanism of Action of D-glyceraldehyde-3-phosphate Dehydrogenase

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A computer approximation with polynomial quotients was used to evaluate from experimental data the dependence of the initial velocity of D-glyceraldehyde-3-phosphate dehydrogenase reaction on the concentration of substrates. The initial velocity values were determined at optimum conditions, over a wide range of substrate concentrations and by interpolating the time curve of enzyme reaction as $t \rightarrow 0$.

A further computer approximation with polynomial quotients, without any implied hypotheses, gave the best fit to the experimental results. The analysis of this final equation shows that two types of catalytic sites may exist.

Due to the complexity of the system, the results are compatible either with the ordered binding or with rapid equilibrium random binding of substrates to each separate, but interacting type of sites. Previous experimental data showing the formation of abortive and dead-end complexes can be interpreted as kinetic effects, inherent in the mechanism. Results at variance with earlier data can be explained by the different experimental conditions.

Introduction

There are a lot of controversial data concerning the mechanism of action of D-glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12). Furfine and Velick (1965) assumed a rapid equilibrium general mechanism in glyceraldehyde-3-phosphate oxidation using arsenate ion (irreversible process) and D,L-GAP as substrate (containing L-GAP, which inhibits the enzyme – cf. Keleti et al., 1973; Tomova et al., 1977). A compulsory ordered mechanism was set forth by Orsi and Cleland (1972) with D,L-GAP as substrate and phosphate ion at a concentration which already inhibits the activity. A cyclic mechanism was presented by Trentham (1971b) and Harrigan and Trentham (1973) but these workers used suboptimal phosphate concentrations, at a pH lower than the optimum, where the conversion of the diol form of GAP into the aldehyde may be rate limiting (Trentham et al., 1969). Under certain experimental conditions the dissociation of NADH seems to be the rate limiting step (Trentham, 1971a; Peczon, Spivey, (1972).

Abbreviations: GAP or G, D-glyceraldehyde-3-phosphate; NAD or N, nicotinamide-adenine-dinucleotide, oxidized; NADH, reduced NAD; P_i or P, inorganic phosphate ion

Keleti and Batke (1965) as well as Tro and Keleti (1974) found a rapid equilibrium partially random AB mechanism (cf. also Trentham 1971a; Harrigan, Trentham 1971, 1973, 1974) with D-GAP, phosphate ion up to the optimal concentration and at optimal pH. The rate limiting step was the phosphorolysis of the 3-phosphoglyceryl enzyme (Keleti, Telegdi, 1959b; Keleti, Batke, 1967).

However, it seems that the present accuracy of determinations (Keleti et al., 1973) does not meet the requirements set by the mathematical formalisms available (Cleland, 1963; Keleti, Batke, 1965; Dalziel, 1969; Wong, Hanes, 1969; Keleti, 1969. 1972). The picture becomes even more complicated if one considers the existence of abortive and dead-end complexes (Nagradova, 1958; Severin, Nagradova 1958; Keleti, Telegdi, 1959a; Ferdinand, 1964; Batke, Keleti, 1968; Ovádi et al., 1972; Keleti, 1973), as well as the activating complexes that involve two substrates per subunit (Batke, Keleti, 1968; Gelb et al., 1970, 1974).

Therefore, to elucidate the real mechanism of action of D-GAP oxidation it was necessary to perform a great number of automated velocity measurements and to develop a new theoretical approach to simulate the kinetic data obtained. These results are presented in this paper.

Materials and methods

Pig muscle D-glyceraldehyde-3-phosphate dehydrogenase, recrystallized four times, was prepared according to Elődi and Szörényi (1956). The concentration of the enzyme was determined spectrophotometrically at 280 nm, using $A_{1\,cm}^{1\,mg/ml} = 1.0$ (Fox, Dandliker 1956).

D-GAP was prepared from its diethylacetal compound (Boehringer, Mannheim. FRG). NAD was obtained from Boehringer (Mannheim, FRG). All other chemicals were purchased from E. Merck, Darmstadt, FRG.

The initial velocity in the steady-state of the enzyme reaction was determined in triethanolamine-HCl buffer (1.0 M, pH 8.56) at 20 °C. The enzyme concentration in the assay mixtures was 0.1 μg/ml. Absorption was measured at 334 nm with a MPS photometer from Vitatron, The Netherlands. A coupled raction rate computer was used to calculate the rate of change every 10 seconds as μmole NADH/(min × mg protein), beginning at the 10th sec after the start and continuing for several minutes. The initial reaction velocity was determined by interpolation. Standard deviation was not greater than 5%.

The substrate and cosubstrate concentrations were kept within the following ranges:

$[P_i]$ 0.415 – 209 mM (7 concentration values)

[NAD] 0.081 – 19.5 mM (7 concentration values)

[GAP] 0.100 – 1.00 mM (5 concentration values)

The experimental dependence of the reaction velocity on the substrate and cosubstrate concentrations was approximated according to Feraudi and Glaser (1977).

Results and discussion

For a reliable experimental determination of the relationship $v = f([P_i], [NAD], [GAP])$, the examination of a relatively wide concentration range of each substrate is crucial. The highest velocity value in the present experiments was 116.9 ± 5.7 (mean $\pm$ standard error) and the lowest was 8.60 ± 2.25 in μmole NADH/(min $\times$ mg enzyme). Representative examples of the results are presented in Figs 1 to 3.

First one must ascertain which algebraic formula fits the experimental data best.* In the steady-state the approximating equation required is the following:

$$v = \frac{\sum_{j=1}^{n} p_j [P_i]^j}{\sum_{i=0}^{m} q_i [P_i]^i} \times \frac{\sum_{h=1}^{v} p'_h [NAD]^h}{\sum_{k=0}^{w} q'_k [NAD]^k} \times \frac{\sum_{x=1}^{r} p''_x [GAP]^x}{\sum_{y=0}^{t} q''_y [GAP]^y}$$

Since n, m, v, w, r and t are unknown, the calculation of the parameters p_j, p'_h, p''_x, q_i, q'_k and q''_y is practically impossible because of the relatively broad

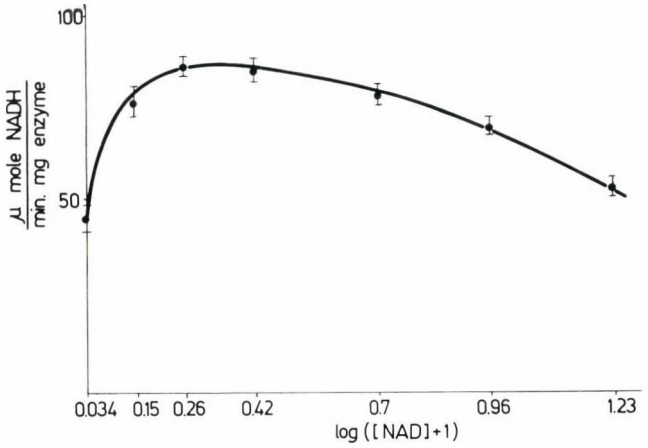


Fig. 1. Saturation of glyceraldehyde-3-phosphate dehydrogenase with NAD. 1.0 M triethanolamine —HCl buffer, pH 8.56, at 20°C. [GAP] = 0.5 mM, $[P_i]$ = 10.7 mM. The experimental data (mean $\pm$ standard error) are fitted to equation (3). On the abscissa one unit was added to the concentration values in mM, to obtain a simpler graphical representation.

* The best fit was that where the residual sum of squares was the smallest. In the steady-state the approximating equation required is the following:

$\sum_{i=1}^{n} (v_i - v_{ij})^2/(n - p_i)$, where v_i and v_{ij} are the measured and calculated velocity values at the *i-th* experimental points, respectively, n is the number of points and p_i is that of the calculated parameters.

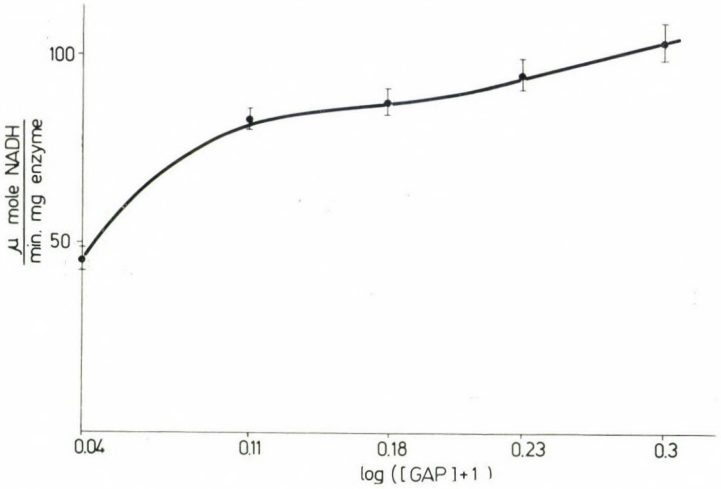


Fig. 2. Saturation of glyceraldehyde-3-phosphate dehydrogenase with GAP. [NAD] = 1.61 mM [P_i] = 10.7 mM. Other conditions see in legend to Fig. 1.

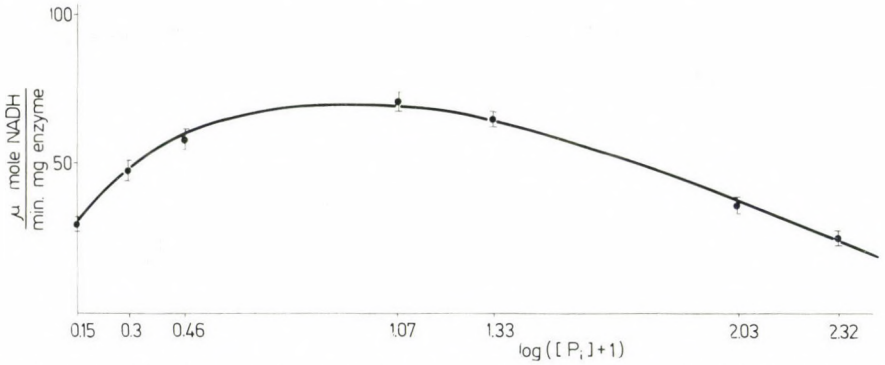


Fig. 3. Saturation of glyceraldehyde-3-phosphate dehydrogenase with P_i. [GAP] = 0.5 mM, [NAD] = 1.61 mM. Other conditions see in legend to Fig. 1.

statistical deviation and the limited number of velocity values. We therefore attempted to estimate n, m, v, w, r and t as follows.

A computer program (Feraudi, Glaser, 1977) was employed to approximate with a polynomial quotient the experimental dependence of the reaction velocity on the concentration of one substrate when the concentration of the other two substrates is kept constant. In this polynomial quotient those values of parameters $p_1 \ldots p_5$, $q_1 \ldots q_5$ were sought which gave the best approximation to the experimental data on the basis of the equation:

$$v = \frac{p_1 s + p_2 s^2 + \ldots + p_5 s^5}{1 + q_1 s + q_2 s^2 + \ldots + q_5 s^5},$$

Table 1

Algebraic form of the polynomial quotient giving the best fit for the dependence of the reaction velocity on the substrate or cosubstrate concentrations

Substrates, the concentration of which was kept constant	Experimental function which was to be approximated	Analytical form of the best fit for each experimental function*
P_i, NAD	$v = f([GAP])$	$v = \frac{p_1 [GAP]}{1 + q_1 [GAP]}$
P_i, GAP	$v = g([NAD])$ **	$v = \frac{p_1' [NAD] + p_2' [NAD]^2}{1 + q_1' [NAD] + q_2' [NAD]^2 + q_3' [NAD]^3}$
NAD, GAP	$v = h([P_i])$**	$v = \frac{p_1'' [P_i] + p_2'' [P_i]^2}{1 + q_1'' [P_i] + q_2'' [P_i]^2 + q_3'' [P_i]^3}$

* Parameters p and q have positive values.

** $[NAD]^3$ and $[P_i]^3$ did not appear in all best fits for all concentrations of substrate that was kept constant.

where s stands for the concentration of the substrate varied. The results of this approximation are presented in Table 1.

When the reaction velocities were approximated as a function of [GAP] only, a quotient of first order polynomials gave the best fit at all substrate concentrations which were kept constant. The polynomial quotients approximating the dependence of the velocity on [NAD] or $[P_i]$ generally contained second order terms, in some cases even third order terms. This is in agreement with previous findings showing inhibition by excess of NAD and P_i (Batke, Keleti, 1968).

The algebraic formulas in Table 1 suggest that the experimental relationship between v and the three substrate concentrations can be approximated by the quotient

$$v = \frac{u_1[GAP](u_1'[NAD] + u_2'[NAD]^2)(u_1''[P_i] + u_2''[P_i]^2)}{(1 + w_1[GAP])(1 + w_1'[NAD] + w_2'[NAD]^2 + w_3'[NAD]^3)(1 + w_1''[P_i] + w_2''[P_i]^2 + w_3''[P_i]^3)} \quad (1)$$

where u-s and w-s are the parameters to be determined.

By the aid of a further computer program (Feraudi, Glaser 1977) an approximation of the experimental data with polynomial quotient (1) was attempted. The results are presented in equation (2), which is the best approximation of the experimental data (terms equalling zero are not given).

$$v = \frac{[GAP][NAD][P_i](r_1 + r_2[P_i] + r_3[NAD][P_i])}{1 + t_1[P_i]^2 + [NAD](t_2[P_i] + t_3[P_i]^2) + [NAD]^2(t_4[P_i] + t_5[P_i]^2) + \\ + [GAP]\{(t_6 + t_7[P_i]) + [NAD](t_8 + t_9[P_i] + t_{10}[P_i]^2 + t_{11}[P_i]^3) + \\ + [NAD]^2(t_{12}[P_i] + t_{13}[P_i]^2)\}} \quad (2)$$

Within the concentration ranges applied several terms in equation (2) never reach significant values compared to the total value of the numerator or the denominator. Hence equation (2) can be replaced by equation (3).

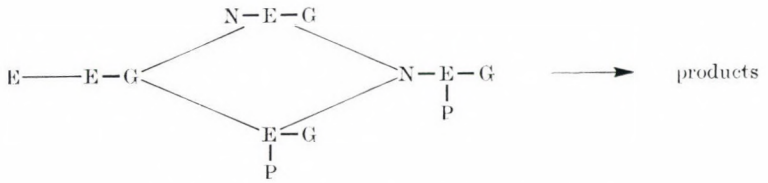
$$v = \frac{[GAP][NAD][P_i](r_1 + r_3[NAD][P_i])}{1 + t_2[NAD][P_i] + t_4[NAD]^2[P_i] + [GAP]\{t_6 + t_7[P_i] + [NAD](t_8 + t_9[P_i]) + \\ + [NAD]^2(t_{12}[P_i] + t_{13}[P_i]^2)\}} \quad (3)$$

Figs 1 to 3 give examples for the fit of equation (3) to the experimental data. The mean deviation of experimental and calculated data is not greater than the standard error.

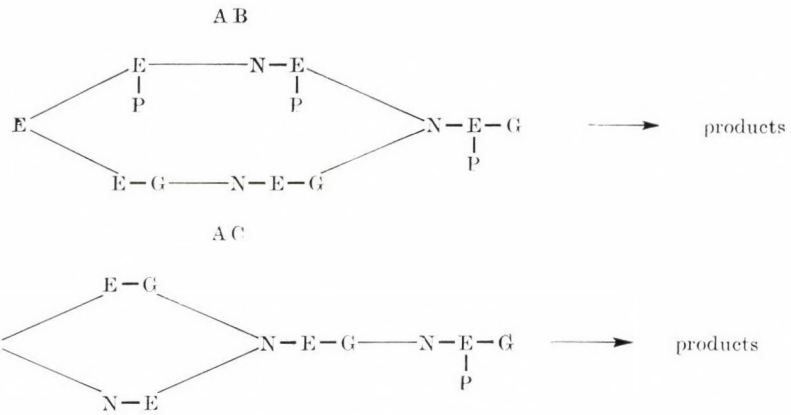
Equation (3) can be regarded as the algebraic expression of the dependence of the reaction velocity on the substrate concentrations. It may serve as a basis for the elucidation of reaction mechanism. To this end one can make either of two assumptions: 1. The catalytic sites are kinetically equivalent; 2. The catalytic sites are kinetically not equivalent but they can be divided into two kinetically different groups (since the second order terms in the numerator and denominator limit the number of these to two).

First let us examine the mechanism on the basis of assumption 1. In the algebraic expression of the experimental relationship the second order is the highest power of [NAD] and [P_i] in the numerator. This feature corresponds to a mechanism involving a sequence of reaction steps in which not more than two molecules of both NAD and P_i can participate. However, the term [GAP] [NAD] [P_i] demands that these steps be distributed over two pathways each containing one step with one NAD and one step with one P_i. It further demands that these two pathways form a cycle where the step with GAP originates from the free enzyme only. In short, the algebraic form of the numerator implies that one part of the reaction mechanism will be similar to Scheme I, where the binding order of NAD and P_i to E-GAP is still not prescribed although equation (3) is not completely equivalent with the steady-state equation of the partially random BC mechanism presented in Scheme I. The nominator further demands that two alternative steps with GAP originate from the free enzyme leading to E-GAP and E′-GAP. However, this speculative interpretation of the algebra of equation (3) finds no experimental support.

The rules of King and Altman (1956) enable one to derive the theoretical velocity equations of mechanisms with different binding orders for two out of the three substrates (Schemes I and II). Table 2 presents the reaction velocity equations for the mechanisms of Schemes I and II.



Scheme I. Partially random BC mechanism



Scheme II. Partially random AB and AC mechanism

Table 2

Algebraic forms of the equations of initial velocity of partially random steady-state mechanisms with three substrates

Partially random AB	$v = \mathrm{GNP}(a+b\mathrm{G}+c\mathrm{N})/(d+e\mathrm{G}+f\mathrm{N}+g\mathrm{P}+h\mathrm{GN}+i\mathrm{GP}+j\mathrm{NP}+k\mathrm{G}^2+l\mathrm{N}^2+m\mathrm{G}^2\mathrm{N}+n\mathrm{G}^2\mathrm{P}+o\mathrm{N}^2\mathrm{P}+p\mathrm{N}^2\mathrm{G}+r\mathrm{NGP}+s\mathrm{G}^2\mathrm{PN}+t\mathrm{N}^2\mathrm{GP})$
Partially random BC	$v = \mathrm{GNP}(a'+b'\mathrm{N}+c'\mathrm{P})/(d'+e'\mathrm{G}+f'\mathrm{N}+g'\mathrm{P}+h'\mathrm{GN}+i'\mathrm{GP}+j'\mathrm{NP}+k'\mathrm{GN}^2+l'\mathrm{GP}^2+m'\mathrm{N}^2\mathrm{P}+n'\mathrm{NP}^2+o'\mathrm{GNP}+p'\mathrm{GN}^2\mathrm{P}+r'\mathrm{GNP}^2)$
Partially random AC	$v = \mathrm{GNP}(a''+b''\mathrm{N}+c''\mathrm{P}+d''\mathrm{GN}+e''\mathrm{GP})/(f''+g''\mathrm{N}+h''\mathrm{P}+i''\mathrm{GN}+j''\mathrm{GP}+k''\mathrm{NP}+l''\mathrm{N}^2+m''\mathrm{P}^2+n''\mathrm{GN}^2+o''\mathrm{GP}^2+p''\mathrm{N}^2\mathrm{P}+r''\mathrm{G}^2\mathrm{N}^2+s''\mathrm{G}^2\mathrm{P}^2+t''\mathrm{GNP}+u''\mathrm{G}^2\mathrm{NP}+v''\mathrm{GN}^2\mathrm{P}+w''\mathrm{GNP}^2+q''\mathrm{G}^2\mathrm{N}^2\mathrm{P}+x''\mathrm{G}^2\mathrm{NP}^2)$

A lack of many terms of the reaction rate equations for partially random mechanisms in equation (3) suggests that the interpretation of experimental data in terms of kinetically equivalent catalytic sites and a steady-state partially random mechanism is inappropriate. Neither of the partially random mechanisms gives under steady-state conditions initial rate equations reducible to equation (3).

Another interpretation of equation (3) is based on the second assumption, i.e. that there are two types of catalytic sites. In this case the algebraic expression can be divided into the sum of two polynomial quotients as shown in equation (4).

$$v=v'+v''=\frac{1}{c_1+c_2\mathrm{G}}\left(\frac{a\mathrm{PNG}}{b_1+b_2\mathrm{N}+b_3\mathrm{P}+b_4\mathrm{PN}}+\frac{a'\mathrm{PNG}}{b'_1+b'_2\mathrm{N}+b'_3\mathrm{P}+b'_4\mathrm{PN}}\right)=$$

$$=\frac{\mathrm{G}[\mathrm{PN}(ab'_1+a'b_1)+\mathrm{PN}^2(ab'_2+a'b_2)+\mathrm{P}^2\mathrm{N}(ab'_3+a'b_3)+\mathrm{P}^2\mathrm{N}^2(ab'_4+a'b_4)]}{(c_1+c_2\mathrm{G})[b_1b'_1+\mathrm{N}(b_2b'_1+b'_2b_1)+\mathrm{P}(b_3b'_1+b'_3b_1)+\mathrm{PN}(b_4b'_1+b'_4b_1+b_3b'_2+b'_3b_2)+}$$
$$+\mathrm{P}^2\mathrm{N}(b_4b'_3+b'_4b_3)+\mathrm{PN}^2(b_4b'_2+b'_4b_2)+\mathrm{P}^2b_3b'_3+\mathrm{N}^2b'_2b_2+\mathrm{P}^2\mathrm{N}^2b'_4b_4] \qquad (4)$$

Some terms in equation (4) do not appear in equation (3). This should be attributed to two facts: first, b_2, b'_2, b_3 and b'_3 are much smaller than b_1, b'_1, b_4 and b'_4, $c_1 < c_2$ and $c_2b_3 < c_1b_2$; second, the experimental data do not allow the resolution of all terms with [GAP] from those without it, because the [GAP] range, which could be used, was too small. According to equation (4) and the assumptions presented above, equation (3) can be regarded as the sum of two algebraically identical velocity equations.

The fit of the experimental data to the theoretical equation (3), (cf. Figs 1 to 3) which can be reconciled with two types of catalytic sites (equation [4]), is in good agreement with previous findings about the functional non-identity of dimers and the half-of-the-sites reactivity of the enzyme (Batke, 1968; Malhotra, Bernhard, 1968; Ovádi et al., 1971; Bernhard, McQuarrie, 1973, Seydoux et al., 1973; Batke et al., 1974; Levitzki, 1974).

The algebraic form of the reaction rate equation applying to each separate type of site can be represented by equation (5).

$$v' = \frac{1}{c_1 + c_2\,\mathrm{G}} \times \frac{a\,\mathrm{PNG}}{b_1 + b_2\,\mathrm{N} + b_3\,\mathrm{P} + b_4\,\mathrm{PN}} \qquad (5)$$

The term $(c_1+c_2\mathrm{G})$ is identical for the two equations, v' and v''. This indicates that the kinetic constants related to GAP are partly identical for the two types of catalytic sites.

The product aPNG in the numerator corresponds to the quaternary complex leading to the product. The terms c_1b_2N, c_1b_3P and b_1c_2G predict the binding of the enzyme to NAD, P_i and GAP to form binary complexes. The term c_1b_4PN predicts the formation of the complex P-E-NAD and c_2b_2NG, c_2b_3PG and c_2b_4PNG predict the formation of the complexes NAD-E-GAP, P-E-GAP and NAD-E-GAP, respectively.
|
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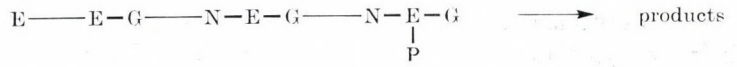
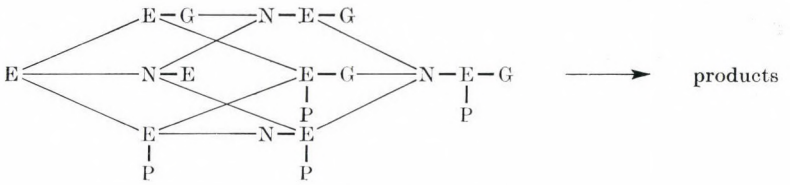


Scheme III. Ordered binding of substrates



Scheme IV. Random mechanism

Equation (5) is perfectly compatible either with an ordered binding of substrates under steady-state conditions (Scheme III) or with a partially random binding (Schemes I and II), or random binding of substrates under pseudo-equilibrium conditions (Scheme IV), to each separate type of sites.

However, the steady-state initial rate equation of the mechanism in Scheme III should not contain in the denominator any significant term including only N. We established with computer simulation, that of all partially random mechanisms in Schemes I and II only the rate requation of the mechanism in which GAP is bound first to the free enzyme in the formation of the productive complex is reducible to equation (5).

The inhibitory effect of excess NAD and P_i (Keleti, Telegdi, 1959a; Batke, Keleti 1968) can be interpreted in the first case (ordered binding) as a result of subunit interactions between the two types of sites due to the fact that the conformation of the enzyme is altered after binding the substrate(s), (Előd i, Szabolcsi, 1959; Listowsky et al., 1965, Keleti, Batke, 1965, Bolotina et al., 1966; Závodszky et al., 1966; Keleti, 1968a). This subunit interaction may manifest itself either in positive or negative cooperativity (Monod et al., 1965; Koshland et al., 1966) or in the association-dissociation of the oligomeric enzyme during catalysis (Kurganov, 1967; Frieden, 1967; Nichol et al., 1967; Keleti et al., 1977; Batke, 1977).

In the second case (random or partially random mechanism), if the quasi-equilibrium condition is not strictly valid, the inhibition and activation by excess substrate may be a kinetic effect, inherent in the mechanism itself (Botts, Morales, 1953; Dalziel, 1957; 1958; Keleti, 1968b; Petterson, 1969a, 1969b).

The present experiments differed from those of Keleti and Batke (1965, 1967) and Tro and Keleti (1974) who found a rapid equilibrium partially random AB mechanism, in that here triethanolamine buffer was used rather than glycine. The latter is known to activate the enzyme according to Tomova et al. (1972, 1974, 1976a, 1976b, 1977). It is possible that the activating effect of amino acids consists in promoting the formation of the EP binary complex thus permitting the formation of product *via* EPN or EPG and EPNG complexes, too.

The main advantage of the method of Feraudi and Glaser (1977) is that no preliminary hypotheses concerning the mechanism of enzyme action are required. Newertheless, the present work shows that in the case of such a complex mechanism even this method fails to decide unequivocally between different possibilities. In a simpler case, e.g. with alcohol dehydrogenase this method was successfully employed (Feraudi et al., 1975, 1977), as it was suitable to demonstrate kinetically the existence of two types of catalytic sites in D-glyceraldehyde-3-phosphate dehydrogenase.

References

Batke, J. (1968) FEBS Letters *2* 81—82
Batke, J. (1977) in preparation
Batke, J., Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 385—395
Batke, J., Keleti, T., Fischer, E. (1974) Eur. J. Biochem. *46* 307—315
Bernhard, S. A., McQuarrie, R. A. (1973) J. Mol. Biol. *74* 73—78
Bolotina, I. A., Volkenstein, M. V., Závodszky, P., Markovits, D. S. (1966) Biokhimiya *31* 649—653
Botts, J., Morales, M. (1953) Trans. Faraday Soc. *49* 696—707
Cleland, W. W. (1963) Biochim. Biophys. Acta *67* 104—137
Dalziel, K. (1957) Acta Chem. Scand. *11* 1706—1723
Dalziel, K. (1958) Trans. Faraday Soc. *54* 1247—1253
Dalziel, K. (1969) Biochem. J. *114* 547—556
Elődi, P., Szabolcsi, G. (1959) Nature *184* 56 (only)
Elődi, P., Szörényi, E. T. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339—350
Feraudi, M., Glaser, W. (1977) Ital. J. Biochem. *26* 22—26
Feraudi, M., Kohlmeier, M., Schmolz, G. (1975) Biochem. Soc. Trans. *3* 1045—1048
Feraudi, M., Kohlmeier, M., Schmolz, G. (1977) Ital. J. Biochem. *26* 12—21
Ferdinand, W. (1964) Biochem. J. *92* 578—585
Fox, J. B., Jr., Dandliker, W. B. (1956) J. Biol. Chem. *221* 1005—1017
Frieden, C. (1967) J. Biol. Chem. *242* 4045—4052
Furfine, C. S., Velick, S. F. (1965) J. Biol. Chem. *240* 844—855
Gelb, W. G., Oliver, E. J., Brandts, J. F., Nordin, J. H. (1970) Biochemistry *9* 3228—3235
Gelb, W. G., Brandts, J. F., Nordin, J. H. (1974) Biochemistry *13* 280—287
Harrigan, P. J. Trentham, D. R. (1971) Biochem. J. *124* 573—580
Harrigan, P. J., Trentham, D. R. (1973) Biochem. J. *135* 695—703
Harrigan, P. J., Trentham, D. R. (1974) Biochem. J. *143* 353—363
Keleti, T. (1968a) Biochim. Biophys. Res. Commun. *30* 185—191
Keleti, T. (1968b) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 247—258
Keleti, T. (1969) in Dévényi, T., Elődi, P., Keleti, T., Szabolcsi, G.: Strukturelle Grundlagen der biologischen Funktion der Proteine. Akadémiai Kiadó, Budapest. pp. 317—522
Keleti, T. (1972) FEBS Lett. *28* 287—288
Keleti, T. (1973) in "Mechanism and Control Properties of Phosphotransferases". Akademie Verlag, Berlin. pp. 253—270
Keleti, T., Batke, J. (1965) Acta Physiol. Acad. Sci. Hung. *28* 195—207
Keleti, T., Batke, J. (1967) Enzymologia *33* 65—79
Keleti, T., Telegdi, M. (1959a) Acta Physiol. Acad. Sci. Hung. *16* 235—241
Keleti, T., Telegdi, M. (1959b) Acta Physiol. Acad. Sci. Hung. *16* 243—255
Keleti, T., Batke, J., Tro, T. Q. (1973) Acta Biol. Med. Germ. *31* 175—179
Keleti, T., Batke, J., Ovádi, J., Jancsik, V., Bartha, F. (1977) Adv. Enzyme Regul. (G. Weber ed.) Vol. 15. Pergamon Press, New York. pp. 233—265

King, E. L., Altman, C. (1956) J. Phys. Chem. *60* 1375—1378
Koshland, D. E., Jr., Némethy, G., Filmer, D. (1966) Biochemistry *5* 365—385
Kurganov, B. I. (1967) Mol. Biol. (USSR) *1* 17—27
Levitzki, A. (1974) J. Mol. Biol. *90* 451—458
Listowsky, I., Furfine, C. S., Betheil, J. J., Englard, S. (1965) J. Biol. Chem. *240* 4253—4258
Malhotra, O. P., Bernhard, S. A. (1968) J. Biol. Chem. *243* 1243—1252
Monod, J., Wyman, J., Changeux, J. P. (1965) J. Mol. Biol. *12* 88—118
Nagradova, N. K. (1958) Biokhimiya *23* 511—522
Nichol, L. W., Jackson, W. J. H., Winzor, D. J. (1967) Biochemistry *6* 2449—2456
Orsi, B. A., Cleland, W. W. (1972) Biochemistry *11* 102—109
Ovádi, J., Telegdi, M., Batke, J., Keleti, T. (1971) Eur. J. Biochem. *22* 430—438
Ovádi, J., Nuridsány, M., Keleti, T. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. *7* 133—141
Peczon, B. D., Spivey, H. D. (1972) Biochemistry *11* 2209—2217
Petterson, G. (1969a) Acta Chem. Scand. *23* 2717—2726
Petterson, G. (1969b) Acta Chem. Scand. *23* 3243—3247
Severin, S. E., Nagradova, N. K. (1958) Dokl. Akad. Nauk SSSR *121* 519—522
Seydoux, F., Bernhard, S. A., Pfenninger, O., Payne, M., Malhotra, O. P. (1973) Biochemistry *12* 4290—4300
Tomova, N., Setchenska, M., Krusteva, N., Christova, Y., Detchev, G. (1972) Z. Pflanzenphysiol. *67* 113—116
Tomova, N., Setchenska, M., Dimitrieva, L., Dimova, O. (1974) Compt. Rend. Acad. Bulg. Sci. *27* 411—414
Tomova, N. G., Dimitrieva, L. M., Dimova, O. B. (1976a) Compt. Rend. Acad. Bulg. Sci. *29* 1665—1668
Tomova, N. G., Ivanova, V. M., Mechev, I. H. (1976b) Compt. Rend. Acad. Bulg. Sci. *29* 1669—1672
Tomova, N., Batke, J., Keleti, T. (1977) Acta Biochim. Biophys. Acad. Sci. Hung. *12* 197—205
Trentham, D. R. (1971a) Biochem. J. *122* 59—69
Trentham, D. R. (1971b) Biochem J. *122* 71—77
Trentham, D. R., McMurray, C. H., Pogson, C. I. (1969) Biochem. J. *114* 19—24
Tro, T. Q., Keleti, T. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 281—294
Wong, J. T. F., Hanes, C. S. (1969) Arch. Biochem. Biophys. *135* 50—59
Závodszky, P., Abaturov, L. B., Varshavsky, Ya. M. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 389—402

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Hydrolysis of Alkyl Ester and Amide Substrates by Papain

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The ratio of the rate constants of acylation of papain with some amino acid ester and amide substrates is unexpectedly low. The contribution to this low ratio by the N-acyl group and the amino acid side chain was studied by measuring the rate constants of substrates containing various acyl groups (benzoyl and benzyloxycarbonyl) and various side chains (glycine, alanine, norleucine, citrulline and arginine). The benzoyl esters were found to be less reactive than the corresponding benzyloxycarbonyl esters, whereas the benzoyl and corresponding benzyloxycarbonyl amides reacted with papain at similar rates. These findings can be explained by the dominance of hydrogen bond formation between the enzyme and amide substrates, which compensates for the less favourable binding of the benzoyl group. It is also apparent from the similar acylation rate constants for norleucine, citrulline and arginine derivatives that the guanidyl group only slightly affects the reaction of arginine derivatives with papain.

Introduction

It has previously been shown (Glazer, Smith, 1971; Asbóth, Polgár, 1977) that a remarkable feature of papain catalysis is that the ratio of hydrolysis rates of an ester and the corresponding amide substrate is much lower (5–250) than that expected from the substantial difference in the chemical reactivity of the ester and amide bonds and that actually observed with serine proteases (10 000–100 000) whose catalytic mechanism is akin to that of papain (Whitaker, Bender, 1965). It has been suggested that the high rate of hydrolysis of an amide, as compared to the corresponding ester, is due to hydrogen bond formation between the $-NH_2$ group of the amide function and the peptide carbonyl group of Asp-158 (Asbóth, Polgár, 1977). Recent X-ray diffraction data support this possibility (Drenth et al., 1976). It is not known to what extent the hydrogen bond, the N-acyl group and the amino acid side chain of the substrate influence this ratio. To answer this question the reaction of papain was investigated by systematically varying the structure of substrate pairs.

Materials and methods

Papain (Sigma Chemical Corporation) was purified on an agarose-mercurial column (Sluyterman, Wijdenes, 1970) and assayed as described previously (Asbóth, Polgár, 1977). The N-benzyloxycarbonyl glycine ethyl ester was gift from

Dr K. Medzihradszky (Institute of Organic Chemistry, L. Eötvös University, Budapest). The N-benzyloxycarbonyl-L-alanine methyl ester and α-N-benzyloxycarbonyl-L-arginine methyl ester hydrochloride were purchased from Vega-Fox Biochemicals, α-N-benzoyl-L-arginine methyl ester hydrochloride from Schuchardt GmbH and α-N-benzoyl-L-citrulline methyl ester from Serva. The N-benzoyl-L-alanine methyl ester was prepared according to Hein and Niemann (1962), N-benzoyl-L-norleucine methyl ester by the method used by Kaplan et al. (1970) for N-benzoyl-L-leucine methyl ester. The N-benzyloxycarbonyl-L-norleucine methyl ester was prepared by reacting N-benzyloxycarbonyl-L-norleucine (Vega-Fox) with diazomethane. All amides except α-N-benzoyl-L-arginine amide hydrochloride (Merck) were prepared from the corresponding ester according to Greenstein and Winitz (1961) in dry methanol containing 30 – 50 equivalents of ammonia.

The kinetic methods for determining the second-order rate constants of acylation have previously been described (Asbóth, Polgár, 1977); in addition the hydrolysis of benzoyl derivatives was also followed by the spectrophotometric method of Schwert and Takenaka (1955) at 253 nm. The enzyme and substrate concentrations used in the experiments are seen in Table 1.

Table 1

Substrate and enzyme concentrations used in the determination of second-order rate constants

			Substrate concentration mM	Enzyme concentration μM
N-benzoyl	L-Alanine	methyl ester	0.2 – 2	0.3 – 1
		amide	0.3 – 1.2	3 – 24
	L-Norleucine	methyl ester	0.06 – 0.6	0.3 – 2.5
		amide	0.25 – 2.5	4 – 6
	L-Citrulline	methyl ester	0.16 – 1.1	1.3 – 5
		amide	0.2 – 0.35	2 – 11
	L-Arginine	methyl ester	0.05 – 0.5	0.4 – 1
		amide	0.17	1.3 – 7
N-benzyloxy-carbonyl	Glycine	methyl ester	0.1 – 2.5	1
		amide	0.5 – 1	20 – 60
	L-Alanine	methyl ester	0.07 – 0.7	0.4 – 0.9
		amide	0.6 – 2	5 – 10
	L-Norleucine	methyl ester	0.04 – 0.4	0.1 – 0.4
		amide	0.02 – 0.2	3 – 7
	L-Arginine	methyl ester	0.03 – 0.55	0.1
		amide	0.1 – 1	4 – 6

Results and discussion

Table 2 shows the second-order acylation rate constants of the reaction of papain with ester and amide substrates containing different N-acyl groups and/or amino acid side chains. It is seen that *a)* the ratio of the rate constants for the benzoyl derivatives is lower than that for the benzyloxycarbonyl derivatives irrespective of the side chain; *b)* the benzoyl esters are less reactive than the corresponding benzyloxycarbonyl esters; *c)* the corresponding benzoyl and benzyloxycarbonyl amides react at similar rates; *d)* the reaction rates of the same derivatives of norleucine, citrulline and arginine differ only slightly. From these results the following conclusions can be drawn.

1. It is the benzoyl group rather than the side chain that is responsible for the low ratio of the rate constants for the benzoyl derivatives. The lower ratio with the benzoyl derivatives is accounted for by a lower reaction rate of the benzoyl esters relative to the corresponding benzyloxycarbonyl esters, as the benzoyl and benzyloxycarbonyl amides react at similar rates. The difference in the rate constants for the benzoyl and benzyloxycarbonyl esters cannot be due to a difference between their inherent reactivities, because the alkaline hydrolyses of benzoyl and benzyloxycarbonyl esters proceed at similar rates as found with both glycine and arginine derivatives (k_{OH} for N-benzoylglycine ethyl ester is 0.9 $M^{-1}s^{-1}$, for N-benzyl-

Table 2

Second-order rate constants of acylation of papain by methyl ester and amide substrates at 25° C, pH 6.3

All reactions were performed in the presence of 2% methanol. Substrate and enzyme concentrations used in the experiments are listed in Table 1.

	N-benzoyl			N-benzyloxycarbonyl		
	ester	amide	$\frac{k_{ester}}{k_{amide}}$	ester	amide	$\frac{k_{ester}}{k_{amide}}$
	$k(M^{-1}s^{-1})$			$k(M^{-1}s^{-1})$		
Glycine	250[a,b]	4[a]	62	900[b,c]	5	180
L-Alanine	430	40	11	2 000	20	100
L-Norleucine	2 400	160	15	13 700	150	92
L-Citrulline	2 400[d]	170	14	—	—	—
L-Arginine	5 600[e]	330[f]	17	30 000	260	115

[a] Data of Asbóth and Polgár (1977)
[b] Ethyl esters
[c] 380 $M^{-1}s^{-1}$; Kirsch and Igelström (1966) 25 °C, pH 6.8
[d] 1808 $M^{-1}s^{-1}$; Williams and Whitaker (1967) 25 °C, pH 6.5
[e] Cf. Sluyterman (1968) and Glazer (1966)
[f] 268 $M^{-1}s^{-1}$; Whitaker and Bender (1965) 25 °C, pH 5.2

oxycarbonyl-glycine ethyl ester 1.0 $M^{-1}s^{-1}$, for α-N-benzoyl-L-arginine methyl ester 1.5 $M^{-1}s^{-1}$ and for α-N-benzyloxycarbonyl-L-arginine methyl ester 1.3 $M^{-1}s^{-1}$). Therefore, we suggest that the interaction of papain, which may involve binding forces and steric factors as well, is less favourable with the benzoyl esters than with the benzyloxycarbonyl esters. This difference is conceivable since the benzyloxycarbonyl group, owing to its similar steric structure, can attach to the binding site designed for the phenylalanyl side chain of the most specific substrates, which the aromatic group of the benzoyl group is unable to do, owing to its shorter link to the amino acid. The unfavourable effect of the benzoyl group, however, does not prevail in the case of amide substrates, in which the hydrogen bond between the amide function and the peptide carbonyl group of Asp-158 fastens the bond to be cleaved. This effect of hydrogen bond formation can account for the similarity of the rate constants found in the benzoyl and benzyloxycarbonyl amide series.

It is noteworthy that in the series of the benzoyl derivatives the glycine compound has an exceptionally high ratio. This suggests that the side chain, even if as small as the methyl group of alanine, affects the binding of the substrate. This is in accord with previous data of Sluyterman (1968) and Brubacher and Glick (1974), who have found that protection against inactivation by several alkylating agents differs markedly with benzoyl glycine ethyl ester and benzoyl arginine ethyl ester.

2. The side chain from glycine to arginine enhances the acylation rates, and this can be explained in terms of a favourable binding of the side chain to the enzyme. Comparison of the rate constants for norleucine, citrulline and arginine derivatives provides information on the binding mode of arginine derivatives. Unlike most substrates of papain, arginine derivatives bear a positive charge which, one would expect, may strongly interact with the carboxylate ion of Asp-158 located close to the catalytic region. Thus, arginine derivatives might be implicated in a binding mode different from that of neutral substrates. However, the small difference in the rate constants for norleucine, citrulline and arginine derivatives is inconsistent with this idea and reveals that neither charge interaction nor H-bond formation (citrulline derivatives) is important in the side chain interaction. It is mainly the nonpolar alkyl side chain, such as in norleucine, that appears to govern the binding in all three cases.

The above results show the role of three factors in positioning the scissile bond: *a)* the H-bond between the amide function and the backbone carbonyl group of Asp-158; *b)* the amino acid side chain, and *c)* the N-acyl group of the substrate. The contribution of factors *a)* and *b)* is advantageous in the present instances, whereas factor *c)* may or may not counteract factors *a)* and *b)*. Thus, the benzoyl group prevents ester substrates to interact with papain most favourably. In the case of amide substrates, this unfavourable effect is abolished owing to the hydrogen bonded amide function. Accordingly, the reaction of the benzoyl derivatives with papain illustrates how catalysis is influenced by a competition between the diverse parts of the same substrate molecule.

References

Asbóth, B., Polgár, L. (1977) Acta Biochim. Biophys. Acad. Sci. Hung. *12* 223—230
Brubacher, L. J., Glick, B. R. (1974) Biochemistry *13* 915—920
Drenth, J., Kalk, K. H., Swen, H. M. (1976) Biochemistry *15* 3731—3738
Glazer, A. N. (1966) J. Biol. Chem. *241* 3811—3817
Glazer, A. N., Smith, E. L. (1971) in The Enzymes, 3rd ed. Boyer, P. D. ed. vol. 3. pp. 501—546 Academic Press, New York and London
Greenstein, J. P., Winitz, M. (1961) Chemistry of the Amino Acids vol. 2. p. 1188 J. Wiley and Sons Inc., New York
Hein, G. E., Niemann, C. (1962) J. Am. Chem. Soc. *84* 4487—4494
Kaplan, H., Symonds, V. B., Dugas, H., Whitaker, D. R. (1970) Can. J. Biochem. *48* 649—658
Kirsch, J. F., Igelström, M. (1966) Biochemistry *5* 783—791
Schwert, G. W., Takenaka, Y. (1955) Biochim. Biophys. Acta *16* 570—575
Sluyterman, L. A. Ae. (1968) Biochim. Biophys. Acta *151* 178—187
Sluyterman, L. A. Ae., Wijdenes, J. (1970) Biochim. Biophys. Acta *200* 593—595
Whitaker, J. R., Bender, M. L. (1965) J. Am. Chem. Soc. *87* 2728—2737
Williams, D. C., Whitaker, J. R. (1967) Biochemistry *6* 3711—3717

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Role of Tetramer ⇄ Dimer Equilibrium in the Dephosphorylation and Activity of Phosphorylase *a*

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Skeletal muscle phosphorylase *a* exists as a tetramer or a dimer depending upon the temperature and protein concentration. The rate of dephosphorylation by phosphorylase phosphatase is very low at 18 °C where phosphorylase *a* exists as a tetramer. Caffeine markedly increases the rate of dephosphorylation of tetrameric phosphorylase *a* at 18 °C but has no effect on the dephosphorylation of the dimeric form. Caffeine also enhances the enzymic activity of phosphorylase *a* at 18°C. The results presented here indicate that caffeine can shift the tetramer ⇄ dimer equilibrium toward the dimeric form at 18°C. This conclusion in supported by sedimentation analyses.

Introduction

Phosphorylase phosphatase converts "active" phosphorylase *a* (EC 2.4.1.1) into "inactive" phosphorylase *b* by cleavage of the phosphate group from a serine residue of phosphorylase *a*. The substrate for phosphorylase phosphatase, phosphorylase *a*, exists as a dimer (mol.wt. 185 000) or a tetramer (mol.wt. 370 000) (Graves, Wang, 1972). We have shown earlier that the tetrameric form of phosphorylase *a* cannot be dephosphorylated by phosphorylase phosphatase (Bot, Dósa, 1971; Bot, Gergely, 1972). At low temperature (15–23 °C) when phosphorylase *a* is present as a tetramer, phosphorylase phosphatase can dephosphorylate the substrate only in the presence of various effectors causing the dissociation of the tetrameric form into the dimeric one. On this basis, it could be argued that the stimulatory effect of glycogen (Bot, Dósa, 1971), glucose (de Barsy et al., 1972; Bailey, Whelan, 1972) and glucose-6-phosphate (Bot, Dósa, 1971; Martensen et al., 1973b) in the phosphatase reaction could be attributed to a dissociation phenomenon.

It is known that AMP inhibits the dephosphorylation of phosphorylase *a* and this inhibition could be due to a change in conformation of dimeric phosphorylase (Martensen et al., 1973a). Caffeine (Varsányi, Bot, 1973) and theophylline (Brandt et al., 1975) may stimulate the activity of phosphorylase phosphatase by abolishing the inhibitory effect of AMP.

The dimeric and tetrameric forms of phosphorylase *a* have very different enzymic activities. It has been demonstrated that tetramer phosphorylase *a* has

only a very slight activity compared with the dimeric form of the enzyme (Metzger, et al., 1967; Huang, Graves, 1970). Glucose, glycogen and hydrolyzed amylose induce an increase of enzymic activity at low temperature promoting the dissociation of the tetrameric form (Wang et al., 1965a, 1965b; Metzger et al., 1967).

The present work shows that caffeine can stimulate the conversion of phosphorylase *a* into *b* at low temperature (18 °C) even in the absence of AMP. The data presented in this paper suggest that the stimulatory effect of caffeine is directly related to the dissociation of tetrameric phosphorylase *a*. These conclusions were reached both on the basis of enzyme activity measurements at low temperature and of data obtained in the analytical ultracentrifuge.

Materials and methods

Rabbit skeletal muscle phosphorylase *a* was prepared from phosphorylase *b* using Mg-ATP and phosphorylase kinase as described by Krebs et al. (1964). Phosphorylase *a* was recrystallized three times in 0.04 M glycerophosphate – 0.01 M mercaptoethanol – 0.002 M EDTA buffer (pH 6.8). Nucleotides were removed from phosphorylase *a* by dialysis against 0.4 M imidazole – 0.04 M citrate buffer at pH 6.8, followed by dialysis against 0.04 M glycerophosphate – 0.01 M mercaptoethanol – 0.02 M EDTA buffer (pH 6.8). The ratio of the absorptions of the enzyme at 260 and 280 nm was 0.55, indicating the absence of nucleotides (Griffiths et al., 1974). Phosphorylase *a* activity was assayed at 30 °C by the procedure of Illingworth and Cori (1953). Specific activity of phosphorylase *a* was 53 units $\times$ mg^{-1} in the presence of 0.016 M glucose-1-phosphate and in the absence of AMP.

Phosphorylase phosphatase was prepared from rabbit skeletal muscle according to the method of Brandt et al. (1975). The enzyme was stored at 0 °C in 40% (w/w) glycerol. Before use the glycerol was dialysed against 0.04 M Tris – 0.01 M mercaptoethanol – 0.002 M EDTA buffer at pH 6.8.

Phosphorylase phosphatase assay: 1.2 mg/ml phosphorylase *a* was incubated in 0.04 M Tris – 0.01 M mercaptoethanol – 0.002 M EDTA buffer (pH 6.8) with phosphorylase phosphatase in the presence or the absence of caffeine. The reaction mixtures were incubated at 30 °C or 18 °C, aliquots (50 μl) were removed at different times of incubation and the reaction was stopped by the addition of 0.1 M NaF – 0.04 M glycerophosphate – 0.002 M EDTA (pH 6.8), to attain a dilution in which the activity of residual phosphorylase *a* could still be measured.

Measurement of the activity of phosphorylase *a* at 18 °C. 2 mg/ml phosphorylase was preincubated without glycogen at 18 °C in the absence and the presence of various concentrations of caffeine. In control experiments 0.5% glycogen was added prior to cooling to 18 °C in order to stabilize phosphorylase *a* in dimeric form. The enzymic activity was measured at 18 °C by adding 1% glycogen and 0.016 M glucose-1-phosphate. The incubation time was 30 or 60 seconds and the reaction was stopped by 5% (final conc.) TCA. After removal

of precipitated protein by filtration the liberated P_i was assayed according to the method of Taussky and Shorr (1953).

Ultracentrifugation. A MOM 3170 analytical ultracentrifuge was used. The movement of the protein boundaries was followed by schlieren optics. The schlieren patterns were analyzed with a microcomparator.

Results

The effect of caffeine on the dephosphorylation of phosphorylase *a* has been investigated. Fig. 1 shows that caffeine did not influence at 30 °C the reaction of phosphorylase phosphatase (a). An identical rate was obtained whether or not caffeine was present. At 18 °C dephosphorylation of phosphorylase *a* did not take place at all. In the presence of caffeine, however, the phosphatase reaction was markedly accelerated at this temperature (b). Caffeine promoted the dephosphorylation of phosphorylase *a* also when it subsequently added to the incubation micture. This indicates a rapid modifying effect of caffeine in "substrate-conformation". Decreasing the caffeine concentration from 5 to 0.5 mM did not influence its stimulatory effect on the phosphatase reaction.

The effect of caffeine on the enzymic activity of phosphorylase *a* has also been investigated. Table 1 shows that at 18 °C, when the enzyme is present as a tetramer, the activity of phosphorylase *a* was very low in comparison with the

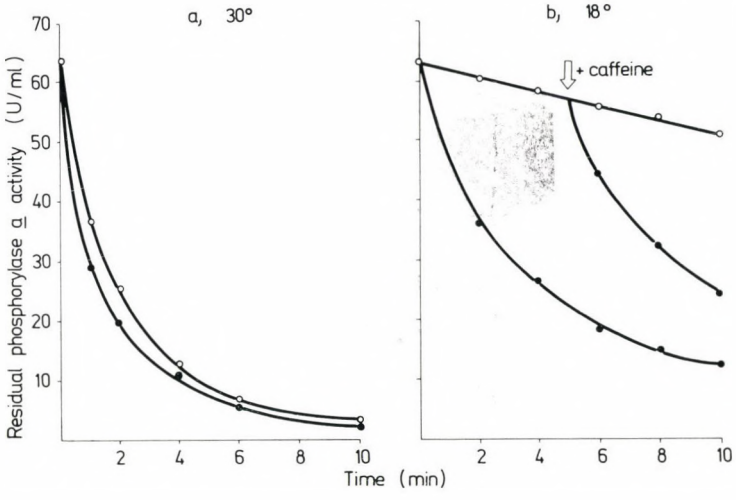

Fig. 1. Interaction of caffeine and temperature in the conversion of phosphorylase *a* into *b*. 1.2 mg/ml (63.6 units) phosphorylase *a* was incubated with 0.1 mg/ml phosphorylase phosphatase in the absence of caffeine (○——○) and in the presence of 5×10^{-3} M caffeine (●——●) at 30°C (a) and 18°C (b). The details of the phosphatase assay are described in Methods. Arrow indicates the adding of 6×10^{-3} M caffeine to the reaction mixture

Table 1

Effect of caffeine on the enzymic activity of phosphorylase a *at 18 °C*

Enzymic activities were measured as described in Methods, concentration of phosphorylase *a* was 2 mg/ml in the assay mixture

Caffeine (mM)	Activity of phosphorylase *a* (U/ml)	
	in the absence of glycogen	in the presence of 0.5% glycogen
none	2.6	11.9
0.5	9.6	11.4
1.0	8.3	10.2
2.5	7.5	8.6
5.0	5.2	7.3

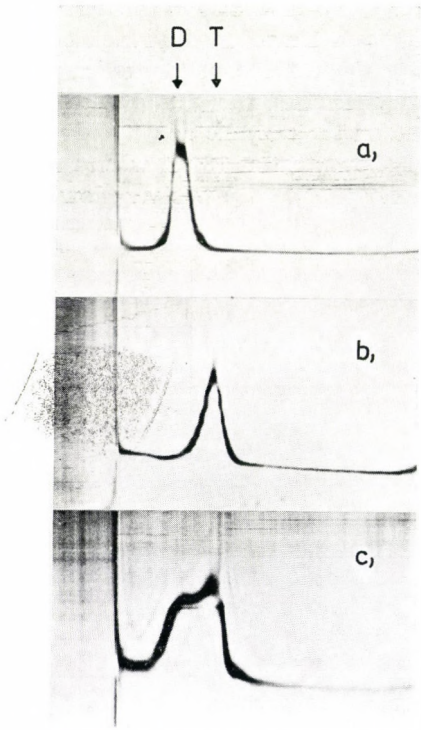

Fig. 2. Effect of temperature and added caffeine on the sedimentation of phosphorylase *a*. Phosphorylase *a* (2 mg/ml) was centrifuged at 50.000 rpm in 0.04 M glycerophosphate — 0.01 M mercaptoethanol — 0.002 M EDTA (pH 6.8). Pictures were taken 18 min after reaching the speed of 50.000 rpm. Direction of sedimentation from left to right. D and T indicate the positions of dimeric and tetrameric forms of phosphorylase *a*. Phosphorylase *a* at 35°C (a) and 18°C (b). Phosphorylase *a* + 5×10^{-4} M caffeine at 18°C (c)

value measured in the presence of glycogen, when the enzyme is present as a dimer.

Caffeine had a biphasic effect on the activity of phosphorylase *a* as can be seen in the results summarized in Table 1. Caffeine alone enhanced the activity of phosphorylase *a* at 18 °C. This stimulatory effect was shown to depend upon the concentration of caffeine. Maximum stimulation was obtained at 0.5 mM caffeine concentration. Higher concentrations of caffeine were less effective.

Caffeine had no stimulatory effect on the activity of phosphorylase *a* in the presence of glycogen. Since glycogen enhances the enzymic activity was a consequence of dimerisation, caffeine can only inhibit this increased activity. The greater the concentration of caffeine the greater was the inhibitory effect on the enzyme.

These observations indicate that caffeine can cause dimerisation of tetrameric phosphorylase *a* at 18 °C. Therefore the effect of caffeine on the sedimentation pattern of phosphorylase was also investigated. Fig. 2. illustrates that phosphorylase *a* can exist as a dimer at 35 °C in the absence or presence of caffeine (a). At 18 °C phosphorylase *a* is a tetramer in the absence of glycogen (b) and the dimeric form appears when caffeine is added (c). Sedimentation coefficients are summarized in Table 2.

Table 2

Influence of caffeine on the sedimentation behaviour of phosphorylase a

Experimental conditions were the same as in Fig. 2. Sedimentation coefficients (S^{20}) are corrected to 20 °C in water

t (°C)	Sample	S^{20}	
		Dimer	Tetramer
35°	Phosphorylase *a*	8.4	none
35°	Phosphorylase *a* + caffeine	8.1	none
18°	Phosphorylase *a*	none	13.8
18°	Phosphorylase *a* + caffeine	8.2	13.4

Discussion

The stimulatory effect of caffeine on the phosphorylase phosphatase reaction is well known (Sutherland, Cori, 1951; Krebs et al., 1964; Stalmans et al., 1970; Varsányi, Bot, 1973). According to recent investigations this stimulatory effect could be due to the cancelling of a conformational change in substrate phosphorylase *a* caused by AMP or other nucleotides (Martensen et al., 1973a). The experiments presented in Fig. 1 show that caffeine stimulates the conversion of phosphorylase *a* into *b* when no nucleotide is present. The stimulatory effect of caffeine at 18 °C indicates its direct interaction with phosphorylase *a*. Since this effect is absent at 30 °C it could be supposed that caffeine shifts tetramer ⇄ dimer equilibrium towards the dimeric form. Caffeine has no stimulatory effect on the phosphatase reaction at 18 °C in the presence of glycogen when phosphorylase *a* exists in dimeric form (not documented) and this fact also supports the above assumption. Furthermore, the dimeric form of phosphorylase *a* in the presence of caffeine could be detected together with the tetrameric form by analytical ultracentrifugation.

It is known that caffeine inhibits the enzymic activity of phosphorylase *a* if the enzyme exist as a dimer (30 °C, low enzyme concentration). The fact that 5×10^{-4} M caffeine enhances the enzymic activity of phosphorylase *a* at 18 °C and at a high enzyme concentration, supports the assumption that caffeine shofts the tetramer ⇄ dimer equilibrium towards the active dimer. It is still unclear whether the shift of the tetramer ⇄ dimer equilibrium is due to a conformational change in the dimeric form or to a direct interaction of the tetrameric form of the enzyme and caffeine.

The results presented here support our earlier observation (Bot, Dósa, 1971; Bot, Gergely 1972) that phosphorylase phosphatase could only dephosphorylate the dimeric form of phosphorylase *a* and not the tetrameric one. Caffeine is a ligand converting tetramer phosphorylase *a* into dimer at low temperature and high protein concentration. Moreover, caffeine is a useful dialysable agent for preventing the crystallization of phosphorylase *a* and it also dissolves phosphorylase crystals at low temperature (4 °C).

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References

Bailey, M. J., Whelan, W. J. (1972) Biochem. Biophys. Res. Commun. *46* 191—197
de Barsy, T., Stalmans, W., Laloux, M., De Wulf, H., Hers, H. G. (1972) Biochem. Biophys. Res. Commun. *46* 183—190
Bot, G., Dósa, I. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. *6* 73—87
Bot, G., Gergely, P. (1972) FEBS Lett. *24* 7—10
Brandt, H., Capulong, Z. L., Lee, E. Y. C. (1975) J. Biol. Chem. *250* 8038—8044

Graves, D. J., Wang, J. H. (1972) in "The Enzymes" (Boyer, B. D., ed.), Vol. 7, pp. 435—482. Acad. Press, New York
Griffiths, J. R., Price, N. C., Radda, G. K. (1974) Biochim. Biophys. Acta *358* 275—280
Huang, C. H., Graves, D. J. (1970) Biochemistry *9* 660—671
Illingworth, B., Cori, G. T. (1953) in "Biochemical Preparations" (Snell, E. E., ed.), Vol. 3, pp. 1—9, John Wiley et Sons, Inc., New-York.
Krebs, E. G., Love, D. S., Bratvold, G. E. Trayser, K. A., Meyer, W. L., Fischer E. H. (1964) Biochemistry *3* 1022—1033
Martensen, T. M., Brotherton, J. E., Graves, D. J. (1973a) J. Biol. Chem. *248* 8323—8328
Martensen, T. M., Brotherton, J. E., Graves, D. J. (1973b) J. Biol. Chem. *248* 8329—8336
Metzger, B., Helmreich, E., Glaser, L. (1967) Proc. Nat. Acad. Sci. USA *57* 994—1001
Stalmans, W., De Wulf, H., Lederer, B., Hers, H. G. (1970) Eur. J. Biochem. *15* 9—12
Sutherland, E. W., Cori, C. F. (1951) J. Biol. Chem. *188* 531—538
Taussky, H. H., Shorr, E. (1953) Acta Biochim. Biophys. Acad. Sci. Hung. *8* 23—31
Varsányi, M., Bot, G. (1973) Acta Biochim. Biophys. Acad. Sci. Hung. *8* 23—31
Wang, J. H., Shonka, M. L., Graves, D. J. (1965a) Biochem. Biophys. Res. Commun. *18* 131—135
Wang, J. H., Shonka, M. L., Graves, D. J. (1965b) Biochemistry *4* 2296—2301

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Chemical Modification of Erythrocytes. Effect on the Velocity of Chromate Uptake

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The velocity of chromate uptake was determined in intact and modified human red blood cells. Modifications included pretreatment with amino- or sulfhydryl reagents, oxidation and enzymic digestion.

The velocity of chromate uptake was strongly inhibited by SITS and DNFB. In both cases maximum inhibition amounted to 90 per cent. This finding is compatible with the assumption that the binding sites for the transported anions, located on the 95 000 dalton m.w. transmembrane protein, also participate in the regulation of the rate of chromate uptake. A fraction of the extracellular chromate rapidly associated with the erythrocytes. The effect of the two reagents differed in that SITS was able to inhibit this step while DNFB was not.

Iodoacetate, iodoacetamide and PCMBS also reduced the rate of chromate uptake by erythrocytes, with a usual maximum inhibition by 75–80 per cent. These observations indicate the participation of SH-group containing components in chromate uptake. The long treatments which were necessary to develop inhibition together with the ineffectiveness of DTNB suggest that the thiol groups in question are not easily acessible from the external side of the membrane.

Pretreatment with periodate inhibited chromate diffusion without altering sulfate transport. The rate constant also decreased when the initial chromate concentration was raised above 0.1 mM. It is supposed that in both cases oxidation is involved in the inhibiton. Sequential treatment with different reagents did not increase further the maximum inhibition achieved by the individual modifications. Sialic acid located on the cell surface although a possible target of oxidative damage does not seem to be related to chromate diffusion because its enzymic removal influenced neither the diffusion itself nor the inhibition caused by periodate.

Introduction

Since the observations of Gray and Sterling (1950) and Sterling and Gray (1950) chromate has been widely applied to label red cells with ^{51}Cr to measure circulating cell volume and life span. Further investigations have shown that the extent of labeling is sensitive to a number of factors, such as the choice of anticoagulant (Necheles et al., 1953), storage (Ebaugh et al., 1953; Schmidt et al.,

Abbreviations used: SITS, 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonic acid; DNFB, 1-fluoro-2,4-dinitrobenzene; DTNB, 5,5'-ditiobis-(2-nitrobenzoic acid); PCMBS, p-chloromercurybenzene-sulfonic acid.

1960) and experimental conditions (Mossman, Ebaugh, 1957). In the cell interior chromium, after reduction to the trivalent form is bound mainly to hemoglobin (Gray, Sterling, 1950) and to some extent to other cell constituents (Prins, 1962; Skrabut et al., 1976). Kinetic measurements have shown that the rate of uptake is determined by transmembrane diffusion rather than by some consecutive events (Ormos, Mányai, 1974). Thus the differences obtained in the extent of labeling may be related in many cases to changes in the parameters of chromate diffusion.

Studies on the mechanism of chloride and sulfate diffusion across the red cell membrane indicate that the transport of anions proceeds largely by a special exchange mechanism with the participation of the 95 000 dalton molecular weight transmembrane protein (Cabantchik, Rothstein, 1974a, b). Chromate diffusion resembles sulfate transport in its pH-dependence and competition with chloride. Its activation energy, however, is much lower, approximately 10 kcal/mole. Moreover it is insensitive to the quality and valency of the bulk anionic components (Ormos, Mányai, 1974) which are known to influence the transport of other anions (Deuticke, 1967; Wieth, 1970). These differences suggest that the mechanism of chromate diffusion cannot be explained by analogy to sulfate transport. Consequently both the wide application of chromate to label red cells and the efforts to understand the function of the plasma membrane in the transport of individual anions make it reasonable to study the molecular mechanism of this particular transport.

In this work the rate of chromate uptake was determined in human red blood cells modified by amino- and sulfhydryl-reagents, oxidizing agents and by digestion of the sialic acid on the cell surface to survey the sites that are involved.

Materials and methods

Blood stored in ACD solution for less than a week was washed three times with the incubation medium. Equilibrium anion distribution was established and then the erythrocytes were preincubated in the presence of the modifiers. Chromate diffusion was started by mixing modified cells and prewarmed medium containing $^{51}CrO_4$ (1–2 μCi pro ml cell suspension). Samples of 0.5 ml were added to 3 ml ice cold medium, centrifuged and the disappearance of the extracellular radioactivity was measured by means of an automatic gamma-counter (Gamma NZ-310). Sulfate transport was measured by the equilibrium exchange of $^{35}SO_4$ (2–5 μCi pro ml cell suspension). Samples were passed through Sephadex G-75 gel columns as described by Till et al. (1972) and ^{35}S activity within the cells was determined as described earlier (Ormos, Mányai, 1974).

Sialic acid was estimated by the thiobarbituric assay method (Warren, 1959). Clostridium perfringens neuraminidase was kindly donated by Dr Hrabák (Semmelweis Medical School, Budapest). Sialic acid digestion of washed cells was performed at 37° and pH 7.4 by adding 0.62 μg enzyme pro ml cells at the

beginning and at the half time of the 6 hour long treatment. Afterwards the cells were washed and used for further modifications and transport measurements.

4-acetamido-4'-isothicoyanato-stilbene-2,2'-disulphonic acid was purchased from BDH; 1-fluoro-2,4-dinitrobenzene from Serva; 5,5'-dithiobis-(2-nitrobenzoic acid) from Fluka; p-chloromercurybenzene sulphonic acid from Sigma. Other chemicals were of reagent grade and were obtained from Reanal, Budapest.

Results

A number of amino-reagents have been shown to inhibit the exchange diffusion of anions by interacting with proper intramembrane sites. The reduction of the velocity of chromate uptake by DNFB and by SITS is shown in Figs 1 and 2, respectively. The cells exposed to DNFB were suspended in a medium that, besides NaCl, contained sucrose as well (see the legend to Fig. 1) to avoid hemolysis. The cells were washed between preincubation and the measurement of chromate uptake. This was necessary to remove the breakdown product of DNFB and to expel unreacted SITS that otherwise would have reduced the extracellular chromate to Cr(III) cation.

Although a great part of SITS is reversibly bound by the erythrocyte membrane, this step seems admissible because simple washing could not remove it (Cabantchik, Rothstein, 1972).

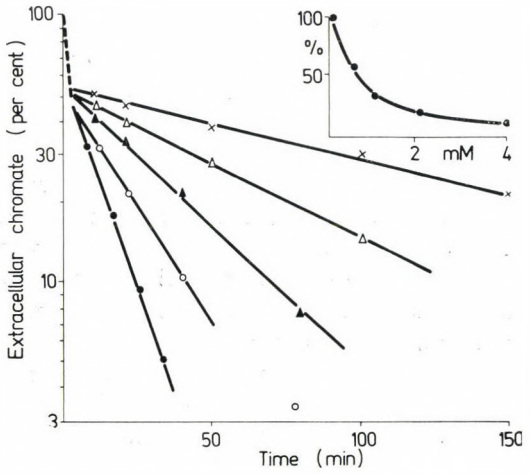

Fig. 1. Inhibition of chromate entry into red cells by DNFB. Treatment: 37°C; 90 min; pH 7.5. Hematocrit: 50 per cent. Medium: 60 mM NaCl, 186 mM sucrose. DNFB dissolved in ethanol was added to the cell suspensions. Final ethanol concentration amounted in all incubation mixtures to 1 per cent. DNFB concentrations: ●, None; ○, 0.5 mM; ▲, 1 mM; △, 2 mM; ×, 4 mM. Chromate uptake by repeatedly washed cells was performed at pH 7.5; 37°C; 25 per cent hematocrit. Initial extracellular chromate concentration: 0.05 mM. Figure inlet: relative rate of chromate diffusion at different DNFB concentrations.

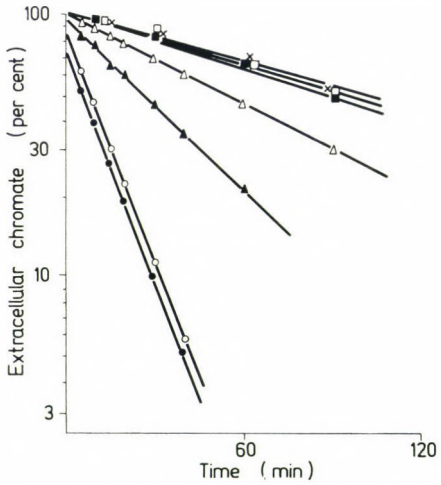

Fig. 2. Inhibition of chromate uptake by SITS. Treatment: 0°C; 60 min; pH 7.4. Hematocrit: 40 per cent. Medium: 142 mM NaCl, 6.25 mM Na-phosphate. Chromate uptake was measured after washing the treated cells: hematocrit: 32 per cent; initial chromate concentration 0.056 mM. Concentrations of SITS in μmoles/ml cell suspension: ●, None: ○, 0.075; ▲, 0.125, △, 0.175: ■, 1.25; □, 2.5; ×, 3.75.

The firm binding of DNFB also allows washing (Poensgen, Passow, 1971). Figs 1 and 2 also show that the decrease of chromate concentration in the extracellular fluid involves a comparatively rapid initial step which is then followed by a lasting process of first order kinetics. SITS was able to inhibit both the rapid association of chromate with the erythrocytes and the consecutive diffusion. DNFB inhibited only the diffusion without affecting the initial step.

The participation of the sulfhydryl-groups of erythrocytes in chromate uptake was tested by PCMBS, iodoacetate, iodoacetamide and DTNB. None of these reagents influenced the initial removal of chromate from the extracellular fluid but they inhibited, with the exception of DTNB, the diffusion step. The development of inhibition needed different lengths of time (Fig. 3a). The maximum inhibition achieved at 2 mM reagent concentration amounted to 75–80 per cent (Fig. 3b). PCMBS is known to increase cation permeability in red cells (Knauf, Rothstein, 1971a). The accompanying hemolysis was decreased by additions of sucrose (see legend to Fig. 3a). The other SH-compounds produced no noticeable hemolysis.

The inhibition of chromate diffusion by SH-blocking agents pointed to the importance of thiol groups in the process. As these ligands submit easily to oxidation we tried to influence chromate movement by oxidizing agents as well. The results in Fig. 4 indicate that in periodate-treated cells chromate uptake was inhibited. Sulfate transport on the other hand was not influenced by periodate. Maximum inhibition of chromate diffusion amounted to 75 per cent at 0.4 mM

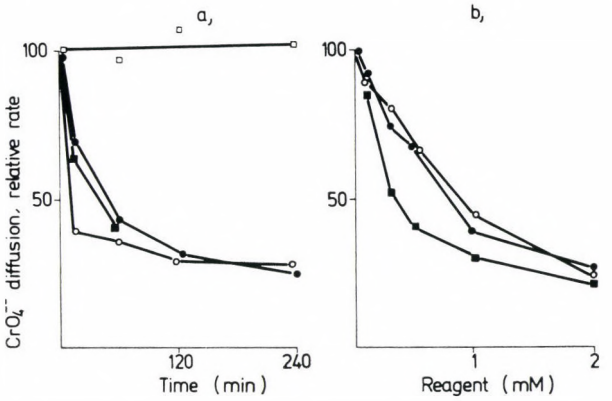

Fig. 3. Inhibition of chromate diffusion by SH-group reagents. Treatment: 37°C; pH 7.4; hematocrit: 40 per cent. *a:* 1 mM reagent *b:* 120 min incubation. Medium: 130 mM KCl, 20 mM Na-phosphate (Fig. 3b PCMBS experiment: + 33 mM sucrose). Reagents: ●, iodoacetate; ○, iodoacetamide; ■, PCMBS; □, DTNB. Initial chromate concentration: 0.057 mM. Ordinate: rate constant for chromate diffusion, per cent of untreated control, Abscissa: Fig. 3a, preincubation time; Fig. 3b. reagent concentration.

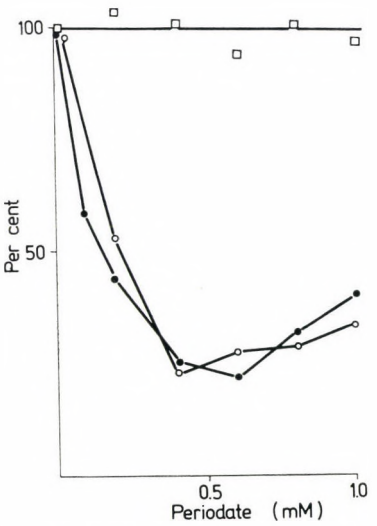

Fig. 4. Effect of sodium periodate on sulfate and chromate transport into red cells. Treatment: 37°C; 30 min; pH 7.4. Hematocrit: 40 per cent. Medium: 135 mM NaCl, 10 mM Na-phosphate, 5 mM Na-sulfate. □, rate of sulfate transport per cent of control; ●, ○, rate constant for chromate diffusion (0.048 mM initial concentration) into intact and neuraminidase digested cells (93±5 per cent removal of the sialic acid) respectively per cent of untreated control.

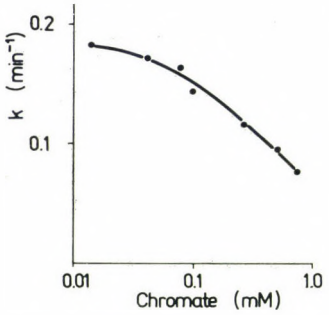

Fig. 5. Dependence of the rate constant for chromate diffusion on the initial extracellular concentration of chromate. Experimental conditions 30°C; pH 7.4; 40 per cent hematocrit. Ordinate: rate constant for chromate diffusion. Abscissa: initial extracellular chromate concentration.

periodate concentration. Under similar experimental conditions potassium bromate at 0.4 mM and 0.8 mM concentrations caused an inhibition of 40 per cent and 55 per cent, respectively. Removal of 93±5 per cent of the sialic acid from the membrane altered neither the rate of uptake of chromate nor its inhibition by periodate.

Chromate is also a potent oxidizing agent. One may therefore expect that an increase in its initial concentration would change as periodate does the rate constant of the diffusion in the same direction. Fig. 5 shows in fact that the velocity constant decreases when the initial chromate level is increased. The change is slight between 0.01 mM and 0.1 mM and becomes pronounced above 0.1 mM.

Table 1

Effect of sequential treatments of red cells on chromate diffusion

Experimental conditions: Medium: 142 mM NaCl, 6.25 mM Na-phosphate; 30 min; 37°C; pH 7.4. Hematocrit: step 1, 40 per cent; step 2, 32 per cent; during chromate uptake, 25 per cent. Initial chromate concentration: 0.029 mM.

Treatment		Inhibition per cent*
1	2	
SITS, 0.5 mM**	—	92.4±1.7
SITS, 0.5 mM**	Iodoacetamide, 2 mM	91.7±1.7
Periodate, 0.4 mM	—	70.0±1.5
Periodate, 0.4 mM	Iodoacetamide, 2 mM	72.4±2.0

* average of triplicates and S.D. of mean
** excess of reagent removed by washing.

None of the treatments described, although significantly reducing the velocity of chromate diffusion caused complete inhibition. Therefore it seemed useful to investigate the effect of a sequential treatment of the cells with different reagents. In Table 1 the inhibitions of chromate diffusion by combined treatments with reagents at concentrations of maximum individual effect are compared. The data show that iodoacetamide did not to a noticeable extent increase the inhibition caused either by SITS or by periodate.

Discussion

The experiments have shown that chromate influx into erythrocytes is regulated by both SITS-sensitive sites and SH-groups.

With intact red cells chromate diffusion proceeds until a nearly perfect uptake because of intracellular chemical reactions. Theoretically a modification may alter either the diffusion rate or the extent of maximum uptake, this latter for instance by impeding the reduction of chromate to its trivalent form and its binding to hemoglobin. In the experiments presented the inhibitors decreased the rate constant rather than the extent of total uptake. This suggests that the modifiers influenced the diffusion mechanism, i.e. cell membrane sites and possible cooperating intracellular molecules.

DNFB and SITS reduced the rate of chromate diffusion with a maximum inhibition of about 90 per cent. The nature of the remaining chromate flux is unknown. It seems, however, of minor importance in comparison with the main transport route. On the basis of this observation one may say that any inhibition that exceeds 10 per cent is related to an alteration of the SITS-sensitive main pathway.

Inhibition of sulfate transport by DNFB (Poensgen, Passow, 1971) and SITS (Knauf, Rothstein, 1971a; Schwoch et al., 1974) takes place in a similar manner as found here for chromate. The similarities strongly support the view that the specific anion binding sites of the 95 000 dalton molecular weight protein also participate in the regulation of chromate uptake. The multiple interactions of these reagents with erythrocytes, however, do not allow definite conclusions. Lately Zaki et al. (1975) reported that on the erythrocyte membrane at least three different DNFB binding sites can be located and that also SITS reacts with more than one population of sites.

Part of the initial chromate content of the medium was rapidly bound by the erythrocytes. An early association of a part of chromate with leukocytes is also known (Lilien et al., 1970). We found that SITS was able to block the association with red cells but DNFB could not prevent it. This result makes it improbable that the anion transport sites, sensitive to both SITS and DNFB, participate in this initial step. Because DNFB reacts with uncharged $-NH_2$ groups (Passow, 1969) and SITS with positively charged groups (Cabantchik, Rothstein, 1972), the initial chromate association may by linked to positively charged, SITS-sensitive

sites independent of anion transport. Chemical separations also showed that after the labeling of red cells, part of the radioactivity still remained in the hexavalent form (Latzkovits, Szentistványi, 1972).

The fraction of chromate bound to the cell surface may also be related to some in vivo observations with labeled red cells. In cell survival studies a loss of part of the radioactivity was observed during the first 24 hours after injection of labeled cells (Mollison, Veall, 1955; Hughes-Jones, Mollison, 1956). This elimination is the consequence of a rapid elution of ^{51}Cr from the surviving cells (Kleine, Heimpel, 1965), indicating that the radiochromium binding strength is not uniform.

Many transport processes across the erythrocyte membrane are regulated by sulfhydryl-groups. In the particular case of anion exchange their participation seemed improbable because SH-reactive compounds did not influence the transport of sulfate, one of the orthodox test anions (Omachi, 1964; Knauf, Rothstein, 1971a). Deuticke (1976) has pointed out that not all anions share this mechanism and that thiol-groups participate in the translocation of some organic anions. Chromate entry into red cells is a further, SH-sensitive, special anion transport phenomenon, because it can be inhibited by PCMBS, iodoacetamide and iodoacetate.

PCMBS and iodoacetate are anionic reagents. Therefore their interaction with the positive anion-binding sites should also be taken into consideration. The modification of chromate uptake in this way, however, can be ruled out for several reasons. First, for the inhibition to develop considerable time was needed. This would not have been the case if electrostatic interaction with sites near the external surface had been involved. Secondly, iodoacetamide, an uncharged molecule, was equally effective. The ineffectiveness of PCMBS on sulfate transport (Knauf, Rothstein, 1971a) also indicates that interaction with the positive anion transport sites is insignificant.

Most of the erythrocyte SH-groups are present in the cell interior, i.e. in hemoglobin and glutathione, whereas the membrane contains less than 5 per cent of the total (Weed et al., 1962). Membrane sulfhydryls form multiple classes according to their accessibility and functional role (Godin, Schrier, 1972; Shapiro et al., 1970; Van Steveninck et al., 1965). As to the location of the SH-groups involved in chromate uptake some information is obtained from data relating to the length of the treatments needed for effective inhibition in the case of PCMBS and the iodinated compounds and to the ineffectiveness of DTNB. Although PCMBS is known to penetrate slowly across the red cell membrane (Knauf, Rothstein, 1971b), a nearly instantaneous inhibition of lactate transfer was observed (Deuticke, 1976). This suggests, together with the inhibitory potency of DTNB, that superficial thiol groups are involved. Osmotic water flow has also been shown to be sensitive to PCMBS (Macey, Farmer, 1970) and DTNB (Brown et al., 1975). A comparison of these observations indicate that the sulfhydryl-groups involved in chromate uptake are identical neither with those participating in lactate transfer nor with the sites of osmotic water movement. The poor accessibil-

ity of the chromate uptake sites suggests that these groups are far from the external membrane surface. Possible SH-compounds that participate in the uptake may be the intracellular solutes in cooperation with the plasma membrane. A further SH-compound is the enzyme glyceraldehyde-3-phosphate dehydrogenase located on the internal membrane surface supposedly in close contact with the 95 000 dalton molecular weight protein (Steck, 1974; Kirkpatrick, 1976). This enzyme has been specifically labeled and inactivated by iodoacetate both in intact cells and isolated membranes (Carraway, Shin, 1972; Shin, Carraway, 1973).

Periodate (Bohn, Brossmer, 1973) and some organic oxidants (Miller, Smith, 1970) have been shown to induce an enhanced loss of K^+ from erythrocytes. In a concentration range of $10^{-4}-10^{-3}$ M effective to increase K^+ leak, periodate did not influence sulfate transport. Chromate diffusion differed from sulfate in this respect as well, since it was inhibited by periodate in this concentration range. According to the investigations of Blumenfeld et al. (1972) the bulk target of periodate oxidation in human erythrocytes is the surface sialic acid. However, since an almost complete removal of the sialic acid altered neither the chromate movement itself nor the inhibition caused by periodate, its participation in chromate uptake is improbable.

The rate constant for the uptake depended on the initial chromate concentration as well. Its diminution was slight in the $0.01-0.1$ mM concentration range, which was therefore selected for most of the experiments, whereas the percentage uptake rapidly decreased at higher concentrations. The value of 0.25 μmoles chromate/ml cell suspension at which the effect of chromate becomes pronounced correspond to 1.5×10^7 CrO_4^{--} ions per cell assuming 3×10^{13} cells/kg dry weight (Dalmark, 1975). At the same time this chromate concentration is close to the recommended upper limit for red cell labeling (Internat. Comm., 1973).

The fact that chromate, if present at concentrations higher than normal, inhibits its own uptake, just as periodate does, may reflect an oxidative damage to the red blood cells.

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References

Blumenfeld, O. O., Gallop, P. M., Liao, T. S. (1972) Biochem. Biophys. Res. Commun. *48* 242
Bohn, B., Brossmer, R. (1973) In: "Erythrocytes, Thrombocytes, Leukocytes" (eds: Gerlach, E., Moser, K., Deutsch, E., Wilmanns, W.), Thieme, Stuttgart, p. 28
Brown, P. A., Feinstein, M. B., Sha'afi, R. I. (1975) Nature *254* 523
Cabantchik, Z. I., Rothstein, A. (1972) J. Membr. Biol. *10* 311
Cabantchik, Z. I., Rothstein, A. (1974a) J. Membr. Biol. *15* 207
Cabantchik, Z. I., Rothstein, A. (1974b) J. Membr. Biol. *15* 227
Carraway, K. L., Shin, B. C. (1972) J. Biol. Chem. *247* 2102

Dalmark, M. (1975) J. Physiol. *250* 39
Deuticke, B. (1967) Pflügers Arch. ges. Physiol. *296* 21
Deuticke, B. (1976) Abstr. FEBS Symp., Biochemistry of Membrane Transport, Zurich, p. 61
Ebaugh, F. G., Emerson, Ch. P., Ross, J. F. (1953) J. Clin. Invest. *32* 1260
Godin, D. V., Schrier, S. L. (1972) J. Membr. Biol. *7* 285
Gray, S. J., Sterling, K. (1950) J. Clin. Invest. *29* 1604
Hughes-Jones, N. C., Mollison, P. L. (1956) Clin. Sci. *15* 207
International Committee for Standardization in Hematology (ICSH) (1973) Brit. J. Haematol *25* 801
Kirkpatrick, F. H. (1976) Life Sci. *19* 1
Kleine, N., Heimpel, H. (1965) Blood *26* 819
Knauf, P. A., Rothstein, A. (1971a) J. Gen. Physiol. *58* 190
Knauf, P. A., Rothstein, A. (1971b) J. Gen. Physiol. *58* 211
Latzkovits, L., Szentistványi, I. (1972) Acta Physiol. Acad. Sci. Hung. *41* 385
Lilien, D. L., Spivak, J. L., Goldman, I. D. (1970) J. Clin. Invest. *49* 1551
Macey, R. I., Farmer, R. E. L. (1970) Biochim. Biophys. Acta *211* 104
Miller, A., Smith, H. C. (1970) Brit. J. Haematol. *19* 417
Mollison, P. L., Veall, N. (1955) Brit. J. Haematol. *1* 62
Mossman, P. L., Ebaugh, F. G. (1957) Federat. Proc. *16* 91
Necheles, T. F., Weinstein, I. M., Le Roy, G. V. (1953) J. Lab. Clin. Med. *42* 358
Omachi, A. (1964) Science *145* 1449
Ormos, G., Mányai, S. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 197
Passow, H. (1969) Progr. Biophys. Mol. Biol. *19* 423
Poensgen, J., Passow, H. (1971) J. Membr. Biol. *6* 210
Prins, H. K. (1962) Vox Sang. *7* 370
Schmidt, H. A. E., Schmitt, H., Keiderling, W., Matthes, M., Feiser, W. (1960) Acta Haematol. *23* 96
Schwoch, G., Rudloff, V., Wood-Guth, I., Passow, H. (1974) Biochim. Biophys. Acta *339* 126
Shapiro, B., Kollmann, G., Martin, D. (1970) J. Cell. Physiol. *75* 281
Shin, B. C., Carraway, K. L. (1973) J. Biol. Chem. *248* 1436
Skrabut, E. M., Catsimpoolas, N., Crowley, J. P., Valeri, C. R. (1976) Biochem. Biophys. Res. Commun. *69* 672
Steck, T. L. (1974) J. Cell Biol. *62* 1
Sterling, K., Gray, S. J. (1950) J. Clin. Invest. *29* 1614
Till, U., Koehler, W., Loesche, W. (1972) Acta biol. med. germ. *28* 51
Van Steveninck, J., Weed, R. I., Rothstein, A. (1965) J. Gen. Physiol. *48* 617
Warren, L. (1959) J. Biol. Chem. *234* 1971
Weed, R., Eber, J., Rothstein, A. (1962) J. Gen. Physiol. *45* 395
Wieth, J. O. (1970) J. Physiol. *207* 581
Zaki, L., Fasold, H., Schuhmann, B., Passow, H. (1975) J. Cell. Physiol. *86* 471

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Effect of Divalent Cations on the Extraction of Nuclear Proteins

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TSCM-(0.01 M Tris-HCl, 0.25 sucrose, 3.8 mM $CaCl_2$, 12 mM $MgCl_2$, pH 7.2) and TS-(0.01 M Tris-HCl, 0.25 M sucrose, pH 7.2)-soluble proteins of isolated rat liver nuclei were analyzed by a two-dimensional polyacrylamide gel electrophoresis method. Light microscopy showed that treatment with the TSCM solution did not alter the morphology of isolated nuclei. After treatment with the divalent cation-free TS solution, the isolated nuclei were swollen; some were disrupted and clumped together. The TSCM solution extracted 20 additional proteins including histones. These results suggest that Ca^{++} and Mg^{++} play an intracellular role in the interactions between nuclear proteins and chromatin.

Introduction

Experiments on isolated nuclei have shown that mono- and divalent cations play a role in the structural and functional organization of nuclear chromatin (Kroeger, 1963; Robert, 1971; Lezzi, 1969; Brasch et al., 1971; Olins, Olins, 1972; Kellermayer, Jobst, 1970, 1971). At 0.2–0.6 M concentration, both mono- and divalent cations disrupt nuclear chromatin structures by dissociation of electrostatic binding of nucleic acids and proteins (Von Hippel, McGhee, 1972; Monneron et al., 1972). Either excessive amounts of cations or loss of cations alter the structure of the nuclear chromatin, especially at neutral pH (pH 7.2–7.5) (Brasch et al., 1971; Olins, Olins, 1972; Kellermayer, Jobst, 1971). During removal of cations isolated nuclei swell and lose structural details even if the osmolarity of the media is maintained (Brasch et al., 1971; Kellermayer, Jobst, 1971). Little is known about the mechanisms of chromatin preservation by Ca^{++} and Mg^{++} at concentrations of 3–12 mM as found in cell nuclei (Langendorf et al., 1961). Their effect on DNA or nuclear structural proteins are not yet defined.

The aim of this work was to study the effects of Ca^{++} and Mg^{++} on the extraction of the nuclear protein with the recently developed two-dimensional electrophoretic technique for protein analysis (Orrick et al., 1973).

The results show that many nuclear proteins are extracted from morphologically intact nuclei by an isotonic sucrose (TSCM) solution containing Ca^{++} and Mg^{++}. Additional proteins, including histones, were extracted from the swollen and disrupted nuclei by the TS solution which lacks divalent cations suggesting that the presence of Ca^{++} and Mg^{++} prevented extraction of nuclear proteins that may maintain the integrity of nuclear chromatin.

Materials and methods

The nuclei were isolated from livers of male Holtzman rats (180–200 g) using a medium of 2.2. M sucrose and 3.3 mM calcium acetate (Busch et al., 1972; Chauveau et al., 1956). The homogenization was carried out with the aid of a Tissumizer (Tekmar SD-45K Super Dispax System) as described earlier (Busch, 1965). After homogenization, the isolated nuclei were centrifuged at 16 000 g for 90 minutes. The pellet was resuspended in fresh isolation medium and pelleted again. The purity of the nuclei was checked by a phase contrast microscope. The nuclei were divided into two parts and incubated in: (1) TSCM solution consisting of 0.25 M sucrose, 3.8 mM $CaCl_2$,12 mM $MgCl_2$, 0.01 M Tris-HCl (pH 7.2) or (2) TS solution consisting of 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.2) at 1 : 10 (V : V), in the cold-room at 4° C with continuous stirring. After incubation for 20 minutes, the nuclei were pelleted at 350 g for 10 minutes to preserve nuclear structure. The pelleted nuclei were treated again with fresh TSCM or TS solution. The supernatants of all extracts were centrifuged at 17 000 g for 15 minutes to sediment particulate elements of the chromatin nuclear sap and nuclear envelope. The extraction and centrifugation steps were repeated three times and the protein content of the nuclear suspension and that of the supernatants were determined by the Lowry method (Lowry et al. 1951).

The three extracts with TSCM or TS solutions were pooled and adjusted to 8 M urea, 0.9 M acetic acid and 5 mM β-mercaptoethanol and then centrifuged for 1 hour at 100 000 g to remove aggregated proteins or RNP particles. The supernatants were concentrated to a protein concentration of 7–10 mg/ml in an Amicon cell using UM-2 Diaflo ultrafiltration membrane (Amicon Inc., Lexington, Mass.). The concentrated samples were dialyzed against a sample buffer containing 10 M urea, 0.9 M acetic acid and 5mM β-mercaptoethanol.

After the nuclei were treated three times with TSCM or TS solution, they were stained in suspension with 0.1% Azure C (Busch, Smetana 1970) for light microscopic analysis.

The proteins were analyzed by the two-dimensional polyacrylamide gel elecrophoretic method. The second gel slab was divided into three (A, B and C) regions and the separated protein spots were marked either with numbers or letters. (Orrick et al., 1973; Yeoman et al., 1973; Kellermayer, Busch, 1973; Kellermayer et al., 1974).

Results

TSCM-treated nuclei had the same light microscopic appearance as that of the control, untreated isolated rat liver nuclei inasmuch as the nucleoli and hetero- and euchromatic structures were equally well preserved (Fig. 1A inset). From these isolated liver cell nuclei, approximately 10% of the total nuclear proteins were extracted by TSCM solution and the nuclei remained well separated even after the third treatment. Twenty-nine dense spots (3, 9 and 17 in the A, B and C regions, respectively) and 28 faint (5, 18 and 5 in the A, B and C regions, respectively), totalling 57 spots, were separated by two-dimensional polyacrylamide electrophoresis (Fig. 1, Table 1). In addition, the density of spots As-r, Bm and B-119 was higher in the TSCM extract than in the TS extract.

The morphology of the isolated cell nuclei was markedly changed after treatment with the TS solution. The nuclei became swollen and some were disrupted; a large number of nuclei clumped together. The nucleoli were generally smaller in size and they disappeared in some cases (Fig. 2A inset).

The TS extracted nuclear proteins accounted for approximately 10% of the total proteins. On the two-dimensional gels of the TS extract, 40 dense spots (7, 11 and 22 in the A, B and C regions, respectively) and 37 faint spots were separated (Fig. 2, Table 1). Of the spots found in the TS extract (Fig. 2B), the GAR and the other histones in spots 1, 2, 3, 17 and 18 in the A region indicate the extraction of chromosomal histone proteins (Orrick et al., 1973; Yeoman et al., 1973). Spot 24 in the A region, spot 7 in the B region and spots 16, 17, 23, 24 in the C region, which are nonhistone nuclear proteins were present in the TS but not in the TSCM extract (Fig. 2 B).

Table 1

Summary of protein spots separated by two-dimensional polyacrylamide gel electrophoretic technique from TSCM and TS extract of rat liver nuclei

"Dense" indicates the protein spots marked on the maps by black and cross-hatched circles and "faint" the protein spots marked on the maps by open or broken circles of Fig. 1B and Fig. 2B

Regions	TSCM extract		TS extract	
	Dense spots	Faint spots	Dense spots	Faint spots
A	3	5	7	12
B	9	18	11	18
C	17	5	22	7
Totals	29	28	40	37
	57		77	

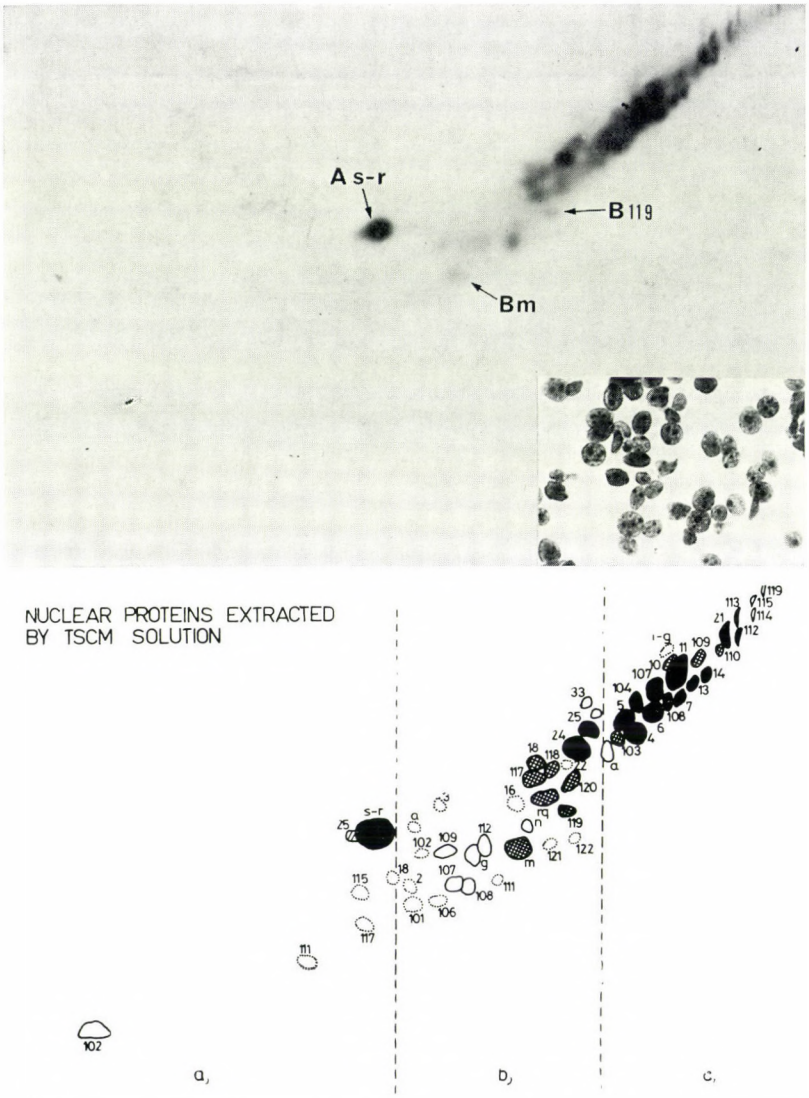
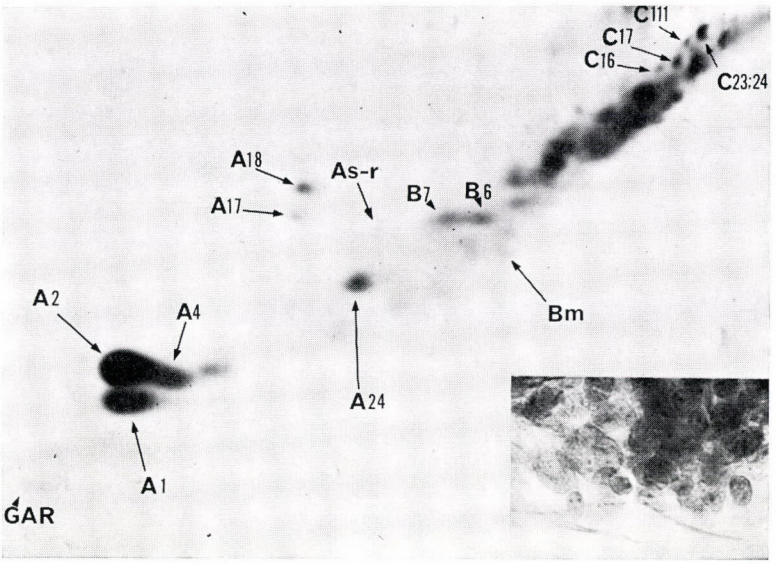


Fig. 1. Two-dimensional polyacrylamide gel electrophoresis of normal rat liver nuclear proteins extracted from isolated nuclei by TSCM solution consisting of 0.25M sucrose, 3.8 mM $CaCl_2$, 12 mM $MgCl_2$ and 0.01 M Tris-HCl (pH 7.2). A. Second dimensional slab gel 400 μg protein was loaded on the first dimension gel tube containing 10% acrylamide and run for 5 hours at 120 V constant voltage in 0.9 M acetic acid. The second dimension was a 12% acrylamide SDS gel. The run was for 14 hours at 50 mA/slab constant amperage. Coomassie blue staining was used for the slab gel. The arrow show the s-r protein complex in the A region, which is much larger in the case of the TSCM extract than in the TS extract. The inset is a light microscopic picture of isolated rat liver cell nuclei treated three times with TSCM solution and stained in suspension with 0.1% Azure C. The nuclei were similar in size and shape to the control, isolated intreated nuclei. The nucleoli and intranuclear chromatin structures were preserved. $\times 600$. B. Diagrammatic representation of the gel pattern shown in Fig. 1A. The system used for the designation of the spots has been described in Materials and methods. The densest spots are black, the less dense spots are cross-hatched, and the faint spots are open or broken circles.



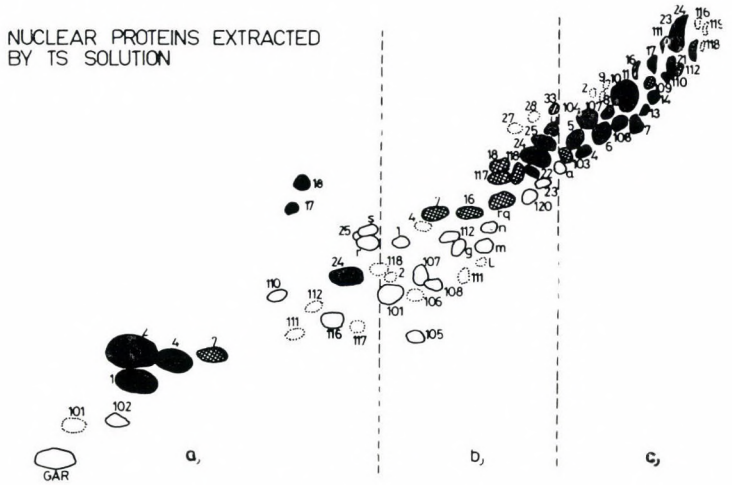


Fig. 2. Two-dimensional electrophoresis of TS-(0.25 M sucrose, 0.01 M Tris-HCl, pH 7.2) extracted proteins. A. Second dimensional slab gel. The conditions were identical to those employed in the experiments shown in Fig. 1A. The numbers indicate the dense spots which were characteristic of the TS extract. The inset is a photograph of nuclei treated three times with TS solution and stained with 0.1% Azure C. ×600. B. Diagrammatic representation of the slab gel pattern shown in Fig. 2A. The numbering system is the same as for Fig. 1B.

Discussion

Some of the diffusible, soluble or cytonucleoproteins are retained in the 2,2 M sucrose, 3,3 mM calcium acetate solution used for isolation of rat liver nuclei. These soluble nuclear proteins were subdivided into two groups. The proteins extracted with TSCM solution were named as unbound, while proteins extracted with solutions (TKCM, TNCM) containing both mono- and divalent cations as "loosely bound" soluble nuclear proteins (Kellermayer et al., 1974).

In the present experiment the proteins were analyzed by a two-dimensional polyacrylamid gel electrophoretic technique. We compared the proteins extracted from isolated rat liver nuclei either with TSCM or TS solution.

(For the composition of the solution see Materials and methods.) The TSCM-soluble nuclear proteins migrated mainly in the high molecular weight B and C regions (Fig. 1). On the other hand, the TS extract contained more proteins that migrated in these regions as well as faster migrating histones. Although the TS extract contained a larger number of the separed spots than the TSCM extract, the amount of the TS-extracted proteins was not greater than that extracted with TSCM. This contradiction might suggest that in the TS solution not only the unbound nuclear proteins were extracted but also several other proteins bound more or less tightly to the chromatin. On the other hand the extraction of the unbound protein from the clumped nuclei with TS solution probably was not so efficient as with the TSCM solution.

The extraction of histones GAR, Al, A2, and A4 ($F2a_1$, $F2a_2$, $F2_b$ and F3 histones) as well as A17 and A18 (F_1 histones) by a nonelectrolyte at the neutral pH of the TS solution was unexpexted because the characteristic salt linkages between the negatively charged groups of the DNA and the positively charged groups of the histone cannot be dissociated under these conditions. The extraction of histones is usually carried out at an ionic strenght of 0.6 or greater or by acid extraction at a pH lower than 2 (Busch, 1965; Hnilica, 1972; Brasch, Setterfield, 1974). The presence of these histones in the 0.25 M sucrose extract may result from fragmentation of some chromatin in the homogenization or extraction steps. These chromatin fragments did not sediment at the 17 000 g centrifugation step but dissociated in 8 M urea, 0.9 M acetic acid and their proteins occurred in the supernatant after centrifugation at 100 000 g. Co-extraction of the higher molecular weight nonhistone proteins with histones by the TS solution suggests that in the cell nuclei, some nonhistone proteins may be bound to histones (Kellermayer et al., 1974).

As mentioned above, Ca^{++} and Mg^{++} at a low concentration (3.8 mM Ca^{++} and 12 mM Mg^{++}), characteristic of the intracellular and intranuclear milieu (Langendorf et al. 1961), prevented the dispersion of chromatin.

The mechanism of the effects of divalent cations in stabilizing the chromatin structures is unknown (Gottesfeld et al. 1974). These results suggest that the chromatin-reserving effects of Ca^{++} and Mg^{++} may result from a prevention of the solubilization of histones and some other nuclear proteins (Von Hahn et

al., 1970) or from binding histones and histone-acidic protein complexes to the DNA. The possibility also exists that Ca^{++} and Mg^{++} exert specific effects on the nuclear membranes.

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References

Busch, H. (1965) Histones and other nuclear proteins. Academic Press, New York
Busch, H., Smetana, K. (1970) The nucleolus. Academic Press, New York
Busch, H., Choi, Y. C., Daskal, I., Inagaki, A., Olson, M. O. J., Reddy, R., Ro-Choi-T. S., Shibata, H., Yeoman, L. C. (1972) Karolinska Symp. No. 5 Stockholm 35—66
Brasch, K., Seligy, V. L., Setterfield, A. (1971) Exptl. Cell. Res *65* 61—72
Brasch, K., Setterfield, G., (1974) Exptl. Cell. Res. *83* 175—185
Chauveau, J., Mould, Y., Roulle r, C. (1956) Exptl. Cell Res. *11* 317—321
Gottesfeld, J., Bagi, G., Bonner, J. (1974) Fed. Proc. *33* 1411
Hnilica, L. S. (1972) The structure and biological functions of histones. CRS Press, Cleveland, Ohio
Kellermayer, M., Busch, H. (1973) Physiol. Chem. Phys. *5* 313—318
Kellermayer, M., Jobst, K. (1970) Exptl. Cell Res *63* 204—207
Kellermayer, M., Jobst, K. (1971) Acta Biol. Acad. Sci. Hung. *22* 321—329
Kellermayer, M., Olson, M. O. J., Smetana, K., Daskal, J., Busch, H. (1974) Exptl. Cell Res. *85* 191—204
Kroeger, H. (1963) Nature *200* 1234—1235
Langendorf, H., Sievert, A., Lorenz, J., Hannover, R., Beyer, R., (1961) Biochem. *335* 273—284
Lezzi, M. (1969) Physiol. Chem. Phys. *1* 447—461
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. *193* 265—275
Monneron, A., Blobel, G., Palade, A. E. (1972) J. Cell Biol. *55* 104—125
Olins, D. E., Olins, A. L. (1972) J. Cell Biol. *53* 715—736
Orrick, L. R., Olson, M. O. J., Busch, H. (1973) Proc. Natl. Acad. Sci. US. *70* 1316—1320
Robert, M. (1971) Chromosoma *36* 1—33
Von Hahn, H. P., Heim, J. M., Eichhorn, A. L. (1970) Biochim. Biophys. Acta *214* 509—519
Von Hippel, P. H., McGhee, J. D. (1972) Ann. Rev. Biochem. *41* 231—300
Yeoman, L. C., Taylor, C. W., Busch, H. (1973) Biochem. Biophys. Res. Commun. *51* 956—966

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Studies on Human Tonsillar Lymphocyte Membranes

II. Biological Properties of Membraneous Glycoprotein Fractions Derived from Human Tonsillar Lymphocytes

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Microsomal and mitochondrial glycoprotein fractions were isolated from human tonsillar lymphocytes. The microsomal glycoprotein was purified by DEAE-Sephadex chromatography. Yielding three subfractions. The different glycoprotein fractions were studied and characterized in two different ways.

The isolated glycoproteins inhibited spontaneous (E) rosette formation. This effect was similar to that exhibited by some immunosuppressive drugs and the inhibitory property was heat-labile.

The microsomal and mitochondrial glycoproteins and the DEAE-Sephadex subfractions of the microsomal glycoprotein inhibited the mitogenic effect of phytohaemagglutinin and concanavalin A. This inhibition was much stronger than the effect of monosaccharide haptens (N-acetyl-D-galactose-amine and -D-glucose) and may be attributed to the lectin-binding property of the glycoprotein fraction.

Introduction

The plasma membranes contain various glycoproteins. These glycoproteins are embedded in the fluid phospholipid bilayer (Singer, Nicolson 1972), and have important functions in transport processes as well as in the immune responses of the cells (as surface antigens or membrane-bound antibodies.) In addition, they serve as receptors for lectins (Marchesi et al, 1972; Pospisilová et al., 1974) and for different hormones (Cuatrecasas, 1974). They are an integral part of the surface membrane and can be solubilized only by detergents, organic solvents, proteolytic enzymes etc.

The erythrocyte membrane has as its major sialoglycoprotein the glycophorin which possesses MN antigenic properties, serves as receptor for influenza virus

Abbreviations used: cAMP, cyclic AMP, adenosine, 3'5'-monophosphate; Con A, concanavalin A, jack bean Concanavalia ensiformis lectin. DEAE, diethyl-amino-ethyl; DNA, deoxyribonucleic acid; LCL, Lens culinaris lectin; LIS, lithium-diiodo-salicylate; PHA, phytohemagglutinin, Phaseolus vulgaris lectin; SDS, sodium dodecyl sulfate; SRBC, sheep red blood cell; WGA, wheat germ agglutinin, Triticum vulgare lectin.

and plant lectins (Marchesi et al. 1972), and also plays a role in rosette formation (Lau, Rosse 1975). Glycophorin contains 60% carbohydrate. The largest part of the membraneous sialic acid can be found in this glycoprotein (Marchesi, Andrews, 1971). The sialic acid component may be responsible for the MN-antigenic property and may form an integral part of the receptor site for influenza virus (Jirgensons, Springer, 1968). This sialoglycoprotein spans the membrane but the localization of carbohydrate units is asymmetric: the sugars are localized exclusively on the external part of the membrane and on the N-terminal part of the protein framework (Marchesi et al., 1972). The amino sugars may be responsible for lectin binding: PHA is bound to N-acetyl-galactosaminyl (Yachnin, 1969) and WGA is bound to the N-acetyl-glucosaminyl groups (Aub et al., 1963, Sharon, Lis, 1972) or the membraneous glycoproteins.

The erythrocyte glycophorin can be solubilized and thus separated from the ghosts by treatment with lithium-diiodo-salicylate (LIS; Marchesi, Andrews, 1971).

LIS is a chaotropic anionic detergent and is more effective in the further purification of glycoproteins than other detergents (Rosai et al., 1971). Glycoprotein fractions can be extracted by solubilization with LIS from other cell types too, such as L 1210 murine leukemic cells (Hourani et al., 1973) or human tonsillar lymphocytes (Hrabák et al., 1977). These solubilized glycoproteins contain high amounts of carbohydrates and serve as binding sites for plant lectins on the cell surface.

Human lymphocyte membraneous glycoproteins solubilized by LIS were investigated for lectin-binding properties and for their capacity of inhibiting E rosette formation. The microsomal glycoprotein (Hrabák et al., 1977) was fractionated by DEAE-Sephadex chromatography and the fractions were tested for lectin-binding property.

Materials and methods

Lithium-diiodo-salicylate was prepared from salicylic acid and ICl and neutralized with LiOH (Woolet, Johnson, 1943). All other reagents were of analytical grade. PHA was purchased from Difco, Con A was a preparation of Pharmacia.

The cells were prepared from tonsils by the method of Piffkó et al. (1970) 10^9-10^{10} cells were used for one experiment. 300–400 mg aceton-dry powder was prepared from 10^9 lymphocytes and 1–1.5 mg glycoprotein could be obtained from 200 mg aceton-dry microsomal powder. The details of the preparation method had been described earlier (Hrabák et al., 1977.)

The microsomal and mitochondrial fractions were prepared according to Hrabák et al. (1977), the plasma membrane was purified from the microsomal fraction by ultracentrifugation (Demus, 1973). The subcellular fractions were

identified on the basis of chemical and enzymatic markers. The lipids of the cellular subfractions were removed by washing with cold acetone.

The glycoprotein was extracted from the lipid-free subcellular fractions by solubilization according to Marchesi and Andrews (1971). The glycoprotein fractions were purified and subjected to DEAE-cellulose chromatography, gel filtration and SDS-polyacrylamide gel electrophoresis as reported earlier (Hrabák et al., 1977). The glycoprotein was found to contain high amounts of various carbohydrates, first of all sialic acid and neutral sugars. The determination of these compounds has also been described previously (Hrabák et al., 1977).

The glycoprotein fraction was chromatographed on a DEAE-Sephadex A 50 column (1.5 × 45 cm) as follows: the solubilized microsomal and mitochondrial fractions (250 μg proteins as determined by the method of Lowry et al., 1951) were applied onto the column in 1 ml of 5 mM Tris-HCl buffer, pH 7.5 and the fractions were eluted by combined ionic-pH gradients and steps.

Gradient I: 5 mM Tris-HCl – 100 mM Tris-HCl (pH 7.5)
Gradient II: 100 mM Tris-HCl (pH 7.5) – 0.5 m Tris-HCl (pH 6.0)
Gradient III: 0.5 M Tris-HCl (pH 6.0) – 2.5 M NaCl (pH 3.0 adjusted by cc. HCl)

After the three gradients two ionic-pH steps were used for the elution of residual proteins: 5 M NaCl (pH 2.0) and 4 N HCl, in a volume of 100 ml each. The ultraviolet absorption of the eluted samples (5 ml) were measured at 230 nm. The fractions were collected, dialyzed against water (3 × 16 h), freeze-dried and stored at 4° C.

The inhibition of spontaneous rosette formation was studied as follows: 100 μl (2×10^8 cells) of a sheep red blood cell suspension in Hanks' medium and 100 μl dialyzed glycoprotein were mixed and incubated for 1 hour at 37 ° C. The glycoprotein was formerly dialyzed against Hanks' medium for 16 hours before adding it to SRBC, to adjust the pH 7.2 – 7.4. After incubation the mixture was stored at 4° C for 1 hour and 100 μl of a human blood lymphocyte suspension (10^7 cells) were added and the mixture was centrifugated immediately.

After centrifugation the mixture was incubated at 4 °C for 1 hour and the rosettes were counted. The criterion of a rosette was the sticking together of at least 4 red blood cells per lymphocyte. The control samples contained 100 μl Hanks' medium instead of the dialyzed glycoprotein. The effect of carbohydrate haptens (glucose, N-acetyl-D-glucosamine, lactose, sialic acid) and that of the heat-inactivated glycoprotein (100° C, 60 min) were tested in the same way.

The inhibitory effect of the glycoproteins on the mitogenic lectins was studied in the following way: 2×10^6 tonsillar lymphocytes were cultured in 1 ml medium containing Eagle basal medium, 10% heat-inactivated human serum, different concentrations of the glycoprotein fractions and of carbohydrate haptens and 100 μl PHA (Difco preparations, dissolved in 5 ml Eagle medium and diluted 100 × with Hanks' medium) or 50 μg Con A. The samples were incubated at 37 °C for 48 hours in sterilized tubes. After 48 hours 3 μCi ^{3}H-thymidine (specific

activity 28.3 Ci/mmol) in 50 μl was added to the cultures and the tubes were incubated for an additional 16 hours at 37 °C. ^{3}H-thymidine incorporation was stopped by adding 1 ml 1 N perchloric acid. The pellet was centrifuged, washed twice with 0.5 N $HClO_4$ at 90 °C for 90 minutes. Hydrolysis was stopped by cooling and the samples were neutralized by the addition of 1 ml 2.5 N KOH. The pellets were centrifuged and 200 μl of the supernatants were solubilized in a toluene-based scintillation cocktail. Radioactivity was measured in a Beckman LS-300 liquid scintillation spectrometer. 500 μl aliquote of the supernatants were used for determining the DNA content of the samples (Burton 1956).

The cytotoxic effect of glycoprotein fractions and subfractions was checked by trypan blue exclusion and by ^{51}Cr-release. The glycoproteins were dissolved in Hanks' medium before the experiments and the pH was adjusted to 7.2–7.4 with 1 N NaOH. Chromium labeling of the tonsillar lymphocytes for the cytotoxicity tests was carried out according to Szabó et al. (1974).

Results

1. DEAE-Sephadex chromatography resulted in the separation of three negatively charged subfractions of the microsomal glycoprotein. The first subfraction (G_1) was eluted by gradient II, the second subfraction (G_2) by gradient III and the residual protein (G_3) in the 4 N HCl step. This elution profile is shown in Fig. 1. The eluted fractions were collected, dialyzed against water (3 × 16 h) and then freeze-dried. The freeze-dried samples were stored at 4 °C before use.

2. Inhibition of rosette formation is shown in Table 2. The glycoprotein fractions isolated by LIS from plasma membranes, microsomes and mitochondria inhibited the spontaneous E-rosette formation of human blood lymphocytes. The inhibitory effect of the mitochondrial fraction is in line with our previous results (Hrabák et al., 1977) according to which the mitochondrial fraction contains membraneous constituents and the glycoprotein derived from membraneous particles may be responsible for the inhibition of the rosette forming capacity. The proportion of rosette forming cells was about 40% and this value was taken as the control. Monosaccharide haptens (glucose, N-acetyl-D-glucosamine), the disaccharide lactose and the polysaccharide dextrane had no effect on rosette formation as shown in Fig. 2. Glycoprotein fractions from the subcellular constituents were heated at 100 °C for 1 h.

This treatment resulted in the aggregation and inactivation of the glycoproteins with respect to the inhibitory effect on E-rosette formation.

3. Inhibition of mitogen activation is shown in Fig. 2 (PHA) and Fig. 3 (Con A). Fig. 3 shows the inhibition of the mitogenic effect of PHA by various concentrations of mitochondrial and microsomal glycoproteins, by the DEAE-Sephadex subfractions as well as by carbohydrate haptens. N-acetyl-D-galactosamine was only hapten which inhibited the mitogenic effect of PHA, but 300 μg of the N-acetyl-D-galactosamine showed a smaller effect than 30 μg glycoprotein

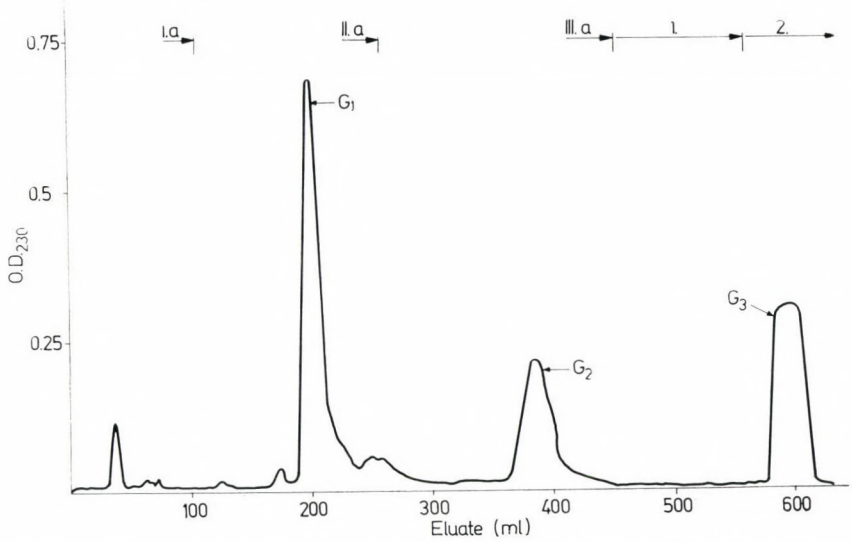

Fig. 1. DEAE-Sephadex chromatography of the microsomal LIS-glycoprotein. G_1; G_2; G_3 = subfractions of the microsomal glycoprotein. I. gradient: 5 mM Tris-HCl – 100 mM Tris-HCl (pH 7.5); II. gradient: 100 mM Tris-HCl (pH = 7.5) – 0.5 m Tris-HCl (pH 6.0); III. gradient: 0.5 M Tris-HCl (pH 6.0) – 2.5 M NaCl (pH 3.0). The gradients were performed by a mixing chamber (100 ml volume). 1. step: 5 M NaCl (pH 2.0) – 100 ml; 2. step: 4 N HCl – 100 ml. The fractions (5 ml volumes) were collected and pooled and after dialysis they were freeze-dried.

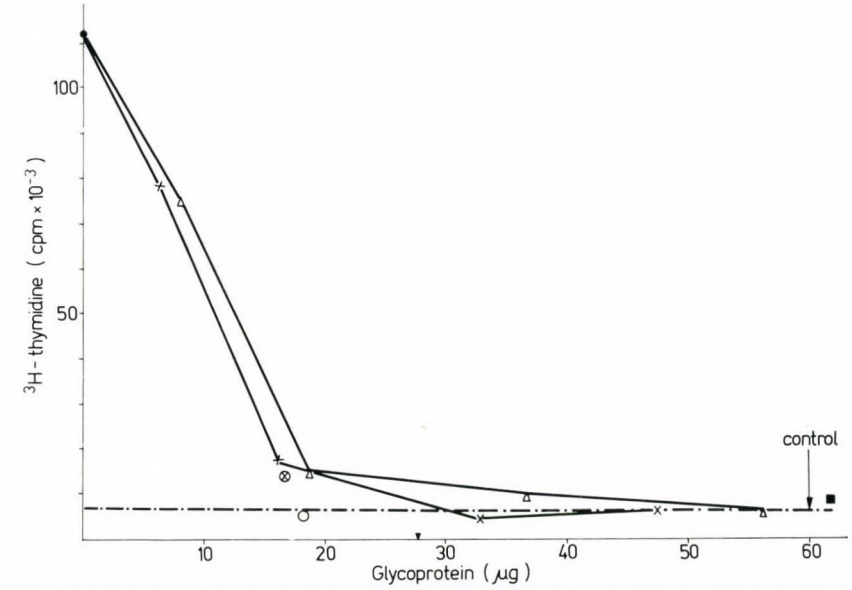

Fig. 2. Effect of glycoproteins on the stimulation of lymphocytes by the mitogen PHA. The control sample (– . – . –) was incubated without any mitogen. Details of the treatment are given in "Materials and methods". ×——× = microsomal LIS-glycoprotein fraction; △——△ = mitochondrial LIS-glycoprotein fraction; ⊕ = 17 µg of subfraction G_2; ○ = 18 µg of subfraction G_1; ▼ = 27.5 µg of the residual protein (G_3); ■ = 300 µg N-acetyl-D-galactosamine.

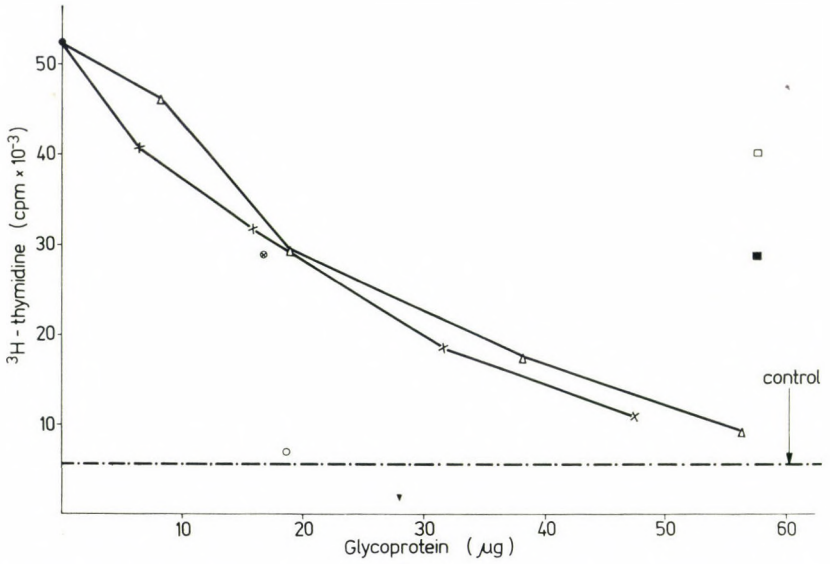

Fig. 3. Effect of glycoproteins on the stimulation of lymphocytes by the mitogen Con A. The control sample (—.—.—) was incubated without any mitogen. Details of the treatment are given in "Materials and methods". ×——× = microsomal LIS-glycoprotein fraction; △——△=mitochondrial LIS-glycoprotein fraction; ⊕ = 17 μg of subfraction G_2; ○= 18 μg of subfraction G_1; ▼ = 27.5 μg of the residual protein (G_3); ■ = 300 μg α-D-glucopyranose; □= 1 mg α-D glucopyranose.

prepared from either the mitochondrial or the microsomal fraction. Other haptens (glucose, N-acetyl-D-glucosamine, N-acetyl-neuraminic acid) were without effect.

The DEAE-Sephadex subfractions also inhibited mitogen activation but the residual (G_3) glycoprotein diminished ^{3}H-thymidine incorporation into the control cells (these cells were not activated by PHA-treatment) too. This subfraction had a strong cytotoxic effect on human tonsillar lymphocytes as shown by trypan blue exclusion and ^{51}Cr-release tests (Table 1). This cytotoxic property was absent in the other glycoprotein fractions.

4. The inhibition of the mitogen activation by Con A can be seen in Fig. 3. Various concentrations of the mitochondrial and microsomal glycoproteins, DEAE-Sephadex subfractions and α-D-glucopyranose were used in the experiments. Each fraction was inhibitory. The effect of glycoprotein fractions and DEAE-Sephadex subfractions was much stronger than that of the glucopyranose hapten: 15–29 μg glycoprotein inhibited the mitogen stimulation to the same extent as 1 mg of glucose. The residual glycoprotein was cytotoxic in these tests as well.

Table 1

Cytotoxic effect of membraneous glycoprotein fractions derived from human tonsillar lymphocytes

Fraction	Living cells* (trypan blue exclusion)	^{51}Cr-release cpm/10^7 cells	^{3}H-Thd incorporation** cpm/10^6 cells
Control	87±5%	1620	3800
Mitochondrial LIS-fraction	80±5%	1580	3430
Microsomal LIS-fraction	81±5%	1610	3450
Subfraction G_1	69±5%	1680	2130
Subfraction G_2	76±5%	1580	2990
Subfraction G_3	5±5%	1970	1040
^{51}Cr-release after disrupting the cells by H_2O	N.D.	4010	N.D.

* 200 cells were counted for determining the percentage of living cells. Each value represents the average of 3 experiments.

** ^{3}H-thymidine incorporation was carried out as described in "Materials and methods" but the time of the incorporation was only 60 min instead of 16 h. The DNA content of 10^6 cells was 6.8 μg as determined by the method of Burton (1956).

Table 2

Effect of glycoprotein fractions on spontaneous rosette formation

Fraction added μg	Number of RFCs*	Percentage of RFCs (% of control)
Control	101±6	100
Plasma membrane glycoportein 50 μg	48±6	47.5
25 μg	67±6	66.4
Microsomal LIS-glycoprotein 54 μg	51±4	50.5
27 μg	59±7	58.5
18 μg	65±6	64.3
Mitochondrial LIS-glycoprotein 29 μg	79±8	78.1
Heat-treated LIS glycoprotein 54 μg	100±4	99.0
Glucose 50 μg	102±4	100.0
N-acetyl-D-glucosamine 50 μg	100±7	99.0
Lactose 50 μg	101±5	100.0

* RFC = rosette-forming cell; 101 rosette-forming cells were found in 250 lymphocytes counted without adding any glycoprotein or other agent.

Discussion

There are several reports concerning to the biochemical, immunological and other properties of human tonsillar lymphocytes, because the tonsil is one of the best source of lymphocytes. Human tonsillar lymphocytes have been reported to have cAMP-dependent histone kinase activity (Faragó et al., 1973, 1975), lysozyme synthesizing capacity (Puskás et al., 1975), and DNA-polymerase activity (Staub, 1974). Plasma membrane fractions of human tonsillar lymphocytes have been isolated by Demus (1973). The cytotoxic activity of human lymphocytes was investigated by Szabó et al. (1974, 1975).

Lithium-diiodo-salicylate – a chatropic agent and anionic detergent – is known to solubilize sialoglycoproteins integrated in plasma membranes and the use of this detergent makes further purification easier than other procedures (Rosai et al., 1971) do. The erythrocyte glycophorin – the first protein solubilized by LIS showed MN-antigenic properties, and contained both influenza virus and lectin receptor sites. Another glycoprotein fraction can be obtained by LIS from L 1210 murine leukemic cells. This fraction consists of four glycoproteins and has the receptor sites for WGA, Con A and LCL (Hourani et al., 1973). LIS-solubilized glycoproteins usually contain large amounts of various carbohydrates and one glycoprotein molecule may serve as receptor site for more than one lectin (Marchesi et al., 1972; Hourani et al., 1973). The receptor site for Co-n A contains α-D-mannopyranoside or α-D-glucopyranoside groups, and that for PHA probably a N-acetyl-D-galactosamine group (Sharon, Lis, 1972).

Our experiments supported the role of a galactosaminyl group as the PHA-receptor sugar (Fig. 3.) as suggested by Yachnin (1969). Four other haptens (glucose, N-acetyl-D-glucosamine, sialic acid and lactose) had no effect on the mitogen stimulation caused by PHA. Inhibition of the mitogenic effect of the lectins PHA and Con A was stronger in the presence of our glycoprotein fraction isolated by solubilization from microsomes and mitochondria derived from human tonsillar lymphocytes than in the presence of monosaccharide haptens. This result suggests that this glycoprotein is responsible for the binding of various lectins on the surface of lymphocytes. Our previous results (Hrabák et al., 1974, Hrabák et al., 1977) also supported this assumption by chemical data: the glycoprotein contained large amounts of carbohydrates. Our fraction contained 278 μg neutral hexose, 122 μg sialic acid and 39.5 μg total hexosamine per mg protein. The hexosamine is present partly in the form of galactosamine probably responsible for the PHA-binding property. The neutral hexose content on the other hand may be responsible for the Con A-binding activity (glucopyranoside or mannopyranoside groups play a role in the binding of Con A).

One of the DEAE-Sephadex subfractions has anomalous characteristics. This subfraction could be eluted from the column only by 4 N HCl and after removing the hydrochloric acid the residual peptide showed a marked cytotoxic effect on human tonsillar lymphocytes. This cytotoxic property has been verified by various methods: the residual protein subfraction reduced DNA-synthesis

in the cells (Fig. 3) caused a marked ^{51}Cr-release from the pre-labelled lymphocytes and decreased the number of living cells measured by the trypan blue exclusion test (Table 1). The other fractions were not cytotoxic to lymphocytes. The reason of this cytotoxicity is difficult to explain: it may be caused by anomalous eluting conditions, because the unfractionated glycoprotein did not show cytotoxicity. It is also possible that some conformational change leads to this cytotoxicity. Perhaps this peptide is localized on the surface of the cells which play a role in cell-cell interactions in the immune reponse.

The E-rosettes are formed by thymus derived lymphocytes and sheep red blood cells. E-rosette formation is characteristic of T-lymphocytes (Bach et al., 1970; Fekete et al., 1972; Bach, Dardenne, 1973; Silveira et al. 1972) and the same cell type can be stimulated by PHA and Con A. Our glycoprotein may be a part of the rosette-forming site on the lymphocyte and may be derived from thymus dependent cells. The rosette inhibition effect is heat-labile and the glycoprotein was shown to aggregate after treatment at 100 °C for 60 minutes. This heat-lability of the glycoprotein suggests that the native form of the polypeptide region is necessary for the inhibition. A similar inhibition can be achieved by immunosuppressive agents (antilymphocytic sera or immunosuppressive drugs, Fekete et al., 1972; Bach, Dardenne 1971). Therefore the inhibition of the rosette forming capacity may indicate that our glycoprotein has some immunosuppressive property.

Recently Lau and Rosse (1975) found that glycophorin played some role in E-rosette formation. It is known that rosette formation can take place at 4 °C but not at 37 °C. It has been suggested that glycophorin is deeply embedded in the lipid matrix at 37 °C. With decreasing temperature SRBC-glycophorin is thought to be exposed at the cellular surface and to serve as a binding site for lymphocytes. After treating the SRBC with neuraminidase the number of rosettes increased (Gilbertsen, Metzgar, 1976). Treatment of the lymphocytes with proteolytic enzymes resulted in a loss of rosette-forming capacity.

The findings support the view that the protein moiety of the rosette receptor plays an important role in rosette formation. It may be supposed that the lymphocytic membraneous glycoprotein has similar properties. The native conformation of its protein moiety may be necessary for its contribution to rosette formation and its function may be temperature-dependent similarly to SRBC-glycophorin.

References

Aub, J. C., Tiesland, C., Lankester, A. (1963) Proc. Natl. Acad. Sci. USA *50* 613
Bach, J. F., Dardenne, M. (1971) Rev. Europ. Etudes Clin. Biol. *16* 770
Bach, J. F., Dardenne, M. (1973) Cell Immunol. *6* 394
Bach, J. F., Delrien, F., Delbarre, F. (1970) Amer. J. Med. *49* 213
Burton, K. (1956) Biochem. J. *62* 315
Cuatrecasas, P. (1974) Annual Rev. Biochem. *43* 169
Demus, H. (1973) Biochim. Biophys. Acta *291* 93
Faragó, A., Antoni, F., Takáts, A., Fábián, F. (1973) Biochim. Biophys. Acta *297* 517

Faragó, A., Romhányi, T., Antoni, F., Takáts, A., Fábián, F. (1975) Nature *254* 88
Fekete, B., Szegedi, Gy., Gergely, P., Szabó, G., Petrányi, Gy. (1972) Acta Med. Acad. Sci. Hung. *29* 327
Gilbertsen, R. B., Metzgar, R. S., (1976) Cell Immunol. *24* 97
Hourani, B. T., Chace, N. M., Pincus, J. H. (1973) Biochim. Biophys. Acta. *328* 520
Hrabák, A., Antoni, F., T. Szabó, M., Merétey, K. (1974) 9th FEBS Meeting Budapest, Abstr. p. 212
Hrabák, A., Antoni, F., T. Szabó, M. (1977) Acta Biochim. Biophys. Acad. Sci. Hung. *12* 245–258
Jirgensons, B., Springer, G. F. (1968) Science *162* 365
Lau, F. O., Rosse, W. F. (1975) Clin. Immunol. Immunopathol. *4* 1
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. *193* 165
Marchesi, V. T., Andrews, E. P. (1971) Science *174* 1247
Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P., Scott, R. E. (1972) Proc. Natl. Acad. Sci. USA *65* 1445
Piffkó, P., Köteles, G., Antoni, F. (1970) Pract. Otorhinolaryng. *32* 305
Pospisilová, J., Haskovec, C., Entlicher, G., Kocourek, J. (1974) Biochim. Biophys. Acta *373* 444
Puskás, M., Staub, M., Antoni, F. (1975) Biochem. Med. *13* 359
Rosai, J., Tillack, T. W., Marchesi, V. T. (1971) Fed. Proc. *30* 453
Sharon, N., Lis, H. (1972) Science *177* 949
Silveise, N. P. A., Mendes, N. F., Tolnai, M. E. A. (1972) J. Immunol. *108* 1456
Singer, D. J., Nicolson, G. L. (1972) Science *175* 720
Staub, M. (1974) 9th FEBS Meeting, Budapest, Abstr. 163.
T. Szabó, M., Hrabák, A., Antoni, F. (1974) J. Nucl. Med. *15* 750
T. Szabó, M., Antoni, F. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. *10* 31
Woolet, J., Johnson, A., (1943) Org. Syntheses Coll. Vol. II. 343
Yachnin, S. (1969) Proc. Natl. Acad. Sci. USA *63* 334

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Effect of Phosphorylation on the Reactivity of the Sulfhydryl Groups of Phosphorylase

(Short Communication)

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It is well-known that SH groups play an important role, directly or indirectly, in the catalytic activity of phosphorylase from skeletal muscle (EC 2.4.1.1). The reactivity of SH groups in phosphorylase *b* and their role in binding substrates (glucose-1-P, P_i^*, glycogen) and activators (AMP) have been studied in detail (Damjanovich, Kleppe, 1966, 1967; Gold, 1968; Kastenschmidt et al., 1968; Kleppe, Damjanovich, 1969). However, surprisingly little information is available as to the SH groups of the partially and totally phosphorylated forms of the enzyme (hybrid phosphorylase and phosphorylase *a*. It has been shown that when applying strong SH reagents, e.g. p-chloromercuribenzoate (Madsen, Cori, 1965) or iodoacetamide (Battel et al., 1968a, b; Avramovic-Zikic et al., 1970), the reactivity of the SH groups of phosphorylase *a* is nearly identical with that of phosphorylase *b*; still, the effect of mild SH reagents is unknown.

Phosphorylase *b* was prepared from rabbit skeletal muscle according to Fischer and Krebs (1958). After three recrystallizations it was transformed to the partially and totally phosphorylated forms (Bot et al., 1974). Before use, mercaptoethanol and AMP were removed from the enzyme solution by gel filtration on a Sephadex G-25 column. The eluting buffer contained 50 mM Tris-HCl (pH 6.8) + 1 mM EDTA. The ratio of the absorptions of the eluted phosphorylase at 260 and 280 nm was 0.56, indicating that the preparation was free of AMP (Fischer, Krebs, 1958).

Protein was determined spectrophotometrically (Appleman et al., 1963).

The reaction of SH groups with DTNB was monitored by measuring the increase in absorption at 412 nm. All reactions were carried out in the presence of 50 mM Tris-HCl (pH 6.8) + 1 mM EDTA, at 25°C. For the calculation of the number of SH groups, the molar extinction coefficient published by Ellman (1959) for thio-2-nitrobenzoic acid, 13 600 $l \times mol^{-1} \times cm^{-1}$, was used.

**Abbreviations:* P_i, inorganic orthophosphate; AMP, adenosine-5-monosphosphate; EDTA, ethylene-diamine-tetraacetic acid disodium salt; DTNB, dithionitrobenzoic acid.

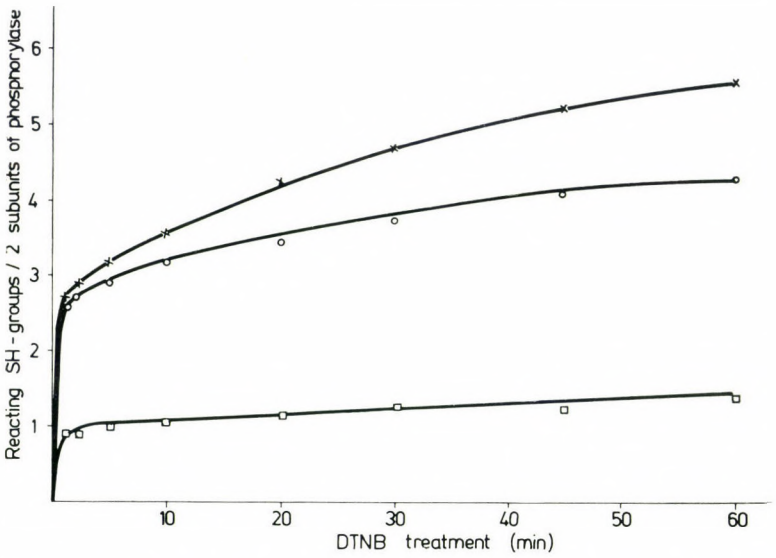

Fig. 1. Number of SH groups reacting rapidly and at medium rate in partially and totally phosphorylated phosphorylases. Reaction mixtures contained 0.5 mM DTNB, 50 mM Tris-HCl (pH 6.8), 1 mM EDTA and 1 mg phosphorylase per ml. Temperature: 25°C. ×——×, phosphorylase *b*; ○——○, partially phosphorylated phosphorylase; □——□ , totally phosphorylated phosphorylase.

Phosphorylase activity was measured according to Illingworth and Cori (1953) in the direction of glycogen synthesis, in the presence of 16 mM glucose-1-P, 1 mM AMP and 1% glycogen.

The reaction with DTNB of the SH group of partially and totally phosphorylated phosphorylases was compared to that of phosphorylase *b*.

It is well-known that two of the 16 SH groups of phosphorylase *b* react rapidly with DTNB while another four react at medium rate (Kleppe, Damjanovich 1969). The reaction of the remaining ten groups is extremely slow; the reactivity of these group in phosphorylated phosphorylases was not studied. Figure 1 shows the increase in the number of SH groups which have reacted with DTNB as a function of time.

Figure 1 clearly indicates that partial phosphorylation of phosphorylase results in a conformational change which does not affect the number of rapidly reacting SH groups, but decreases the number of SH groups reacting at medium rate.

After total phosphorylation of phosphorylase, the number of rapidly reacting SH groups decreases to approximately half of the initial value, while the SH groups reacting at medium rate become completely inaccessible to DTNB.

The reaction of SH groups – especially of those reacting at medium rate – with DTNB considerably decreases the activity of phosphorylase *b*. This

Table 1

Effect of DTNB on the activity of partially and totally phosphorylated phosphorylases

The reaction mixture contained 1 mg enzyme per ml 50 mM Tris-HCl (pH 6.8), 1 mM EDTA and 0.5 mM DTNB. The final volume was 2 ml and the temperature 25°C. Aliquots of 50 μl were withdrawn at various times during incubation, diluted 50-fold with 40 mM Tris-HCl (pH 6.8) buffer and their activity was immediately measured according to Illingworth and Cori (1953).

Time of incubation with DTNB (min)	Activity (%)		
	phosphorylase *b*	partially phosphorylated phosphorylase	totally phosphorylated phosphorylase
0	100	100	100
2	100	71	80
10	86	62	79
20	71	50	79
30	53	48	78
45	27	41	80
60	11	38	79

effect was compared with the change in the activity of partially and totally phosphorylated phosphorylases brought about by DTNB treatment (Table 1).

It has been shown (Kleppe, Damjanovich, 1969) that binding of DTNB to the rapidly reacting SH groups of phosphorylase *b* does not decrease the activity of the enzyme; it is only the reaction of the groups reacting at medium rate that brings about such a decrease. As it is shown in Table 1, the binding of DTNB to the rapidly reacting SH groups of partially and totally phosphorylated phosphorylases already results in a decrease of activity (2 min values). Further incubation of the partially phosphorylated enzyme causes a further decrease of activity, the extent of which is smaller than in the case of phosphorylase *b*, as the partially phosphorylated enzyme contains less SH groups reacting at medium rate (Fig. 1). In the case of totally phosphorylated phosphorylase no further decrease of activity is observed after the binding of DTNB to the rapidly reacting SH groups, in accordance with the fact that the SH groups reacting at medium rate are inaccessible to DTNB in this form of the enzyme.

References

Appleman, M. M., Yunis, A. A., Krebs, E. G., Fischer, E. H. (1963) J. Biol. Chem. *283* 1358–1361

Avramovic-Zikic, O., Smillie, L. B., Madsen, N. B. (1970) J. Biol. Chem. *245* 1558–1565

Battel, M. L., Smillie, L. B., Madsen, N. B. (1968a) Can. J. Biochem. *46* 609–615

Battel, M. L., Zarkadas, C. G., Smillie, L. B., Madsen, N. B. (1968b) J. Biol. Chem. *243* 6202–6209

Bot, G., Kovács, E. F., Gergely, P. (1974) Biochim. Biophys. Acta *370* 70—74
Damjanovich, S., Kleppe, K. (1966) Biochim. Biophys. Acta *122* 145—149
Damjanovich, S., Kleppe, K. (1967) Biochem. Biophys. Res. Comm. *26* 65—70
Ellman, G. L. (1959) Arch. Biochem. Biophys. *82* 70—77
Fischer, E. H., Krebs, E. G. (1958) J. Biol. Chem. *231* 65—71
Gold, A. M. (1968) Biochemistry *7* 2106—2115
Illingworth, B., Cori, G. T. (1953) in Biochemical Preparations (Snell, E. E. ed) *3* 1—9. John Wiley et Sons, Inc., New York
Kastenschmidt, L. L., Kastenschmidt, J., Helmreich, E. (1968) Biochemistry *7* 3590—3607
Kleppe, K., Damjanovich S. (1969) Biochim. Biophys. Acta *185* 88—102
Madsen, N. B., Cori, C. F. (1956) J. Biol. Chem. *223* 1055—1065

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A Full Stochastic Description of the Michaelis-Menten Reaction for Small Systems

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A method is presented to solve the Komogorov-equations for the stochastic model of the Michaelis-Menten reaction. The results are given for the case when only one enzyme molecule is involved in the reaction and can be extended to the case when a few enzyme molecules react. The important differences between the results of stochastic and deterministic treatment are emphasized, and their possible biological implications are discussed. Beside the exact solution of the time course of the irreversible reaction also the equilibrium is described for the reversible reaction. The method provides means for studying other biologically important reactions assuming stochastic behaviour. A comparison is made also with the steady state approximation.

Introduction

Since Delbrück (1940) first used the theory of stochastic processes to approach the kinetics of a reaction of autocatalytic type, and of biological interest, there appeared papers from time to time in the literature by different authors attempting to give a stochastic description of a variety of reactions important in biochemistry. The authors had to cope with considerable mathematical difficulties, especially when complex mechanisms involving elementary steps of second order (e.g. the Michaelis-Menten mechanism) were considered. It was inferred in several papers and has become a widely accepted view that the expectation value obtained by the rather cumbersome methods, and — when really computed — distorted by a series of approximations, differs only slightly from the solutions of the differential equations stemming from the deterministic model. Moreover, the coefficient of variation i.e. the relative standard deviation tends to zero as the number of reacting molecules tends to infinity. As in the case of test tube reactions the number of molecules is generally larger than 10^{10}, the condition for the variance to be neglected is fulfilled. It was roughly concluded therefore that it is an unnecessary complication of the biochemists' work to use a stochastic approach (Heyde, Heyde, 1969, 1971), Only few people were against this view, realizing that a small number effect, or heterogeneity effect should be taken into consideration when systems — not too rare in biochemistry — are investigated which comprise only a small number of at least one of the reactants (Stuart, Branscombe, 1971; Smeach, Smith, 1973). This situation can be the consequence of compartmentalization.

One could think for instance of a specific sequence of DNA that may be present only in a single copy in one cell, or of hydrogen ions of which only one or two can be found at the phsiological pH in a subcellular compartment (Friedrich, 1974). An E. coli cell contains on the average about 20 lac repressor protein molecules, but cells with much less of them should exist in a not absolutely homogeneous population (Stuart, Branscombe, 1971). From a functional point of view a membrane unit can be considered independent of the other thousands of units in the same membrane and the kinetics of the enzymes being present in it in a very limited number duly deserve the elaboration of suitable stochastic models. Indeed, for membrane bound enzymes this work has partly been done (Smeach, Smith, 1973; Smeach, Gold, 1975a, b,).

The aim of the present paper is to solve the stochastic model of the frequently examined Michaelis-Menten mechanism not in the complicated general case, but for small systems including one or few enzyme molecules. We wish to emphasize the important deviation from the deterministic model. The position of the equilibrium in the reversible case is also examined.

Time course of the irreversible reaction

a) *The model*

In this section we first specify a stochastic model for the Michaelis-Menten reaction

$$E + S \leftrightharpoons C \rightarrow E + P \qquad (I)$$

where *E*, *S*, *C* and *P* stand for enzyme, substrate, enzyme-substrate complex and product molecules, respectively. This model does not differ significantly from that given by Bartholomay (1962) and later by Jachimowski et al. (1964). It is even restricted in the sense that it permits only the participation of one enzyme molecule. This crucial simplification, which is, however, justifiable for several biologically important systems, allows us to give an exact solution of the differential equations which will emerge. Furthermore, this solution consists of finite sums of exponential functions. It is by no means complicated, and lends itself easily to comparative studies with the deterministic solution.

The basic assumptions are as follows:

1. We investigate a cell compartment of sufficiently small volume that contains one enzyme molecule, and several substrate molecules. The number of substrate molecules is not limited.

2. At a given moment the enzyme molecule may be free, or complexed with one substrate molecule.

3. The enzyme-substrate complex may dissociate into free enzyme and substrate or enzyme and product molecules.

4. All the substrate molecules act in a stochastically independent and identical manner.

5. The product molecules can neither undergo chemical transformation nor influence the interaction between the enzyme and a substrate molecule.

6. The compartment considered is independent of its environment or the similar compartments inside or outside the cell.

Now we define a stochastic vector process $\{(\xi(t), \zeta(t); 0 \leq t\}$ which is a discrete state, continuous time Markov process with stationary transition probabilities, where the random variable $\xi(t) = 0$, if the enzyme is complexed, and $\xi(t) = 1$ if it is free; the random variable $\zeta(t)$ denotes the number of substrate molecules at a given time, and its value can vary over all the natural numbers from 0 to S_0. The model is completely specified by the infinitesimal transition probabilities (Table 1).

We assume that all the transitions occurring in the interval $(t, t+\Delta t)$, except those given in Table 1, are of probability o (Δt). The probability that more than one transition takes place in the interval $(t, t+\Delta t)$ is also o (Δt). The probabilities of transitions to state (e, s), where $e < 0$, $e > 1$, $s < 0$ or $s > S_0 - 1 + e$ are zero.

We can easily see that state (1,0) is absorbing. So we defined a finite state Markov process with an absorbing state accessible from any other state. Thus, the process has a unique limiting distribution, namely

$$\lim_{t\to\infty} P(e, s; t) = \begin{cases} 1, & \text{if } e = 1,\ s = 0, \\ 0 & \text{otherwise.} \end{cases}$$

(See the Appendix and Arányi, 1976).

So we obtained the not too astonishing result, that, in the limit, our model gives the same result as the deterministic one: after infinitely long time all the substrate molecules are transformed to product molecules and the enzyme is recovered.

Table 1

Infinitesimal transition probabilities

Reaction	Transition	Probability
$E + S \xrightarrow{k_1} C$	$(e, s) \to (e-1, s-1)$	$k_1 \cdot e \cdot s \cdot \Delta t$
$C \xrightarrow{k_{-1}} E + S$	$(e, s) \to (e+1, s+1)$	$k_{-1}(1-e)\,\Delta t$
$C \xrightarrow{k_2} E + P$	$(e, s) \to (e+1, s)$	$k_2\,(1-e)\,\Delta t$

b) *The time course of the stochastic process*

The absolute probability functions can be determined from the Kolmogorov-equations (1)

$$\frac{\mathrm{d}\,\mathrm{P}(e,s;t)}{\mathrm{d}t} = k_{-1}(2-e)\,\mathrm{P}(e-1,s-1;t) + k_2(2-e)\,\mathrm{P}(e-1,s;t) + \tag{1}$$

$$+ k_1(e+1)\,(s+1)\,\mathrm{P}(e+1,s+1;t) - [k_1 e s + (k_{-1}+k_2)\,(1-e)]\,\mathrm{P}(e,s;t)$$

$$(e = 0,1;\ \mathrm{P}(e,s;t) \equiv 0, \quad \text{if} \quad e < 0,\ e > 1,\ s < 0 \quad \text{or} \quad s > \mathrm{S}_0 - 1 + \mathrm{e})$$

through the generating functions. Since the infinitesimal transition probability corresponding to the reaction

$$\mathrm{E} + \mathrm{S} \rightarrow \mathrm{C}$$

is proportional to the number of enzyme as well as of substrate molecules, the partial differential equation to be solved will contain a second order derivative, and cannot be solved exactly (Bartholomay, 1962).

One can define, however, marginal generating functions (Smeach, Smith, 1973) as follows:

$$G_e(z,t) = \sum_{s=0}^{\mathrm{S}_0-1+e} z^s \mathrm{P}(e,s;t) \quad (e = 0,1;\ t \geq 0)$$

Equation (1) can be transformed into a system of partial differential equations for the marginal generating functions by multiplying by z^s and summing over s.

$$\frac{\partial G_e(z,t)}{\partial t} = k_1(e+1)\,\frac{\partial G_{e+1}(z,t)}{\partial z} - k_1\,e\,z\,\frac{\partial G_e(z,t)}{\partial z} -$$

$$- (k_{-1}+k_2)\,(1-e)\,G_e(z,t) + (k_2 + k_{-1})\,z(2-e)\,\mathrm{G}_{e-1}(z,t) \quad (e = 0,1) \tag{2}$$

A simple substitution reveals that the following pair solves equation (2) for a more detailed treatment of equation (2) (see Arányi, 1976)

$$G_0(z,t) = \bar{\Gamma}\,\exp\left[-\frac{k_{-1}}{k_1}(z-1)\right]\exp(-k_2 t) + \bar{\bar{\Gamma}}\,\frac{k_{-1}+k_2}{k_{-1}z+k_2}\exp\left[-(k_1+k_2)\,t\right] +$$

$$+ \sum_{i=1}^{2}\sum_{n=0}^{\infty}\Gamma_i^{(n)}\left[\frac{k_2 - (k_2 + \lambda_i^{(n)})\,z}{-\lambda_i^{(n)}}\right]^{q_n} e^{\lambda_i^{(n)} t};$$

$$G_1(z,t) = \Gamma^{(-1)} - \bar{\Gamma}\,\exp\left[-\frac{k_{-1}}{k_1}(z-1)\right]\exp(-k_2 t) - \bar{\bar{\Gamma}}\exp\left[-(k_{-1}+k_2)\,t\right] -$$

$$- \sum_{i=1}^{2}\sum_{n=0}^{\infty}\Gamma_i^{(n)}\left[\frac{k_2 - (k_2 + \lambda_i^{(n)})\,z}{-\lambda_i^{(n)}}\right]^{q_n+1} e^{\lambda_i^{(n)} t} \tag{3}$$

where

$$\lambda_i^{(n)} \neq -k_2\,, \quad q_n = -\frac{\lambda_i^{(n)2} + (k_{-1}+k_1+k_2)\,\lambda_i^{(n)} + k_1 k_2}{k_1(k_2+\lambda_i^{(n)})}\,, \quad i = 1,\ 2$$

The constants Γ can be determined from the initial conditions:

$$G_0(1, t) + G_1(1, t) = 1 \qquad \text{(a)}$$

$$G_0(z, 0) \equiv 0 \qquad \text{(4) (b)}$$

$$G_1(z, 0) = z^{S_0} \qquad \text{(c)}$$

The conditions (4) are of a deterministic type.

Assuming that the solutions $G_e(z,t)$ to (2) subject to (4) are true generating functions, i.e. are polynomials of finite degree in z, it can be shown that the summation contains a finite number of terms only, and $\bar{\Gamma} = \bar{\bar{\Gamma}} = 0$, if $k_{-1} \neq 0$. The q's are integers, $0 \leq q_n \leq S_0 - 1$, $(q_n = n)$ and the λ's are the roots of the equations

$$\lambda^2 + [k_{-1} + k_1(n + 1) + k_2]\,\lambda + k_1 k_2(n + 1) = 0 \quad (n = 0, 1, \ldots, S_0 - 1) \qquad (5)$$

All the λ's are different negative real numbers. If we take stochastic initial conditions instead of (4) (b) and (c), i.e. $G_0\,(z,0)$ and $G_1\,(z,0)$ are polynomials in z of degrees less than $S_0 - 1 + e$, the solution pair still would contain a finite number of terms only, but of course, the Γ's would be different.

The absolute probabilities can be calculated from the generating functions according to equation (6):

$$P(e, s; t) = \frac{1}{s!} \frac{\partial^s G_e(z, t)}{\partial z^s}\bigg|_{z=0} \qquad (6)$$

Consequently the absolute probabilities themselves are finite sums of exponential functions. They of course, are solutions of the Kolmogorov-equations with the initial conditions

$$P(e, s; 0) = \begin{cases} 1, \text{ if } e = 1, s = S_0 \\ 0 \text{ otherwise.} \end{cases} \qquad (7)$$

The linear algebraic equation system for $\Gamma_i^{(n)}$ obtained from (4) has a unique solution (its determinant is nonvanishing). These parameters, together with $\bar{\Gamma} = \bar{\bar{\Gamma}} = 0$ permit us to calculate $P(e,s;t)$ functions that satisfy the Kolmogorov equation system. As the state space is finite, the latter has a unique solution that can be obtained by means of marginal generating functions.

c) *Comparison with the deterministic model*

The differential equations serving as a deterministic model to reaction (I) – assuming $E_0 = 1$ – are as follows:

$$\begin{aligned} \frac{d[E]}{dt} &= -k_1\,[E]\,[S] + (k_{-1} + k_2)\,(1 - [E]) \\ \frac{d[S]}{dt} &= -k_1\,[E]\,[S] + k_{-1}(1 - [E]) \end{aligned} \qquad (8)$$

where brackets mean concentrations. For the sake of comparison the fixed reaction volume can be taken unity so that concentrations or molecule numbers are expressed by the same value. The initial conditions are

$$[\mathrm{E}](0) = 1, \quad [\mathrm{S}](0) = \mathrm{S} \tag{9}$$

No exact solution to system (8) subject to conditions (9) can be found. It comes from the nature of the deterministic model that – in contrast to the stochastic one – it does not become simpler when the number of reacting molecules is diminished, say to one molecule each. It can easily be shown, as pointed out by many people, that stochastic means are subject to differential equations very similar to (8) with the initial conditions (9). The only difference is that at the right hand sides $\mathrm{E}(\xi \cdot \zeta)$ occurs instead of $\mathrm{E}(\xi)\,\mathrm{E}(\zeta)$. (Here E ( ) means expected values. As $|\,\mathrm{E}(\xi \cdot \zeta) - \mathrm{E}(\xi)\mathrm{E}(\zeta)\,| \leq \mathrm{D}(\xi)\mathrm{D}(\zeta)$, D ( ) means standard deviation, the deterministic solution approaches the stochastic means as $\mathrm{D}(\xi)\mathrm{D}(\zeta)$ tends to zero. The differences between them may become large when the standard deviations are relatively large numbers. The relative difference vanishes as the number of substrate (or in the general case of enzyme) molecules tends to infinity.

Figures 1–4 serve the comparison. The deterministic solutions were obtained by a computer program using the Runge-Kutta approximation. As $S_0 = 1$ was assumed, the marginal generating functions are

$$\begin{aligned} G_0(z,t) &= [k_1/(\lambda_1^{(0)} - \lambda_2^{(0)})]e^{\lambda_2^{(0)}t} - [k_1/(\lambda_1^{(0)} - \lambda_2^{(0)})]\, e^{\lambda_1^{(0)}t} \\ G_1(z,t) &= 1 - [k_1/(\lambda_1^{(0)} - \lambda_2^{(0)})]\,\{[k_2 - (k_2 - \lambda_2^{(0)})\,z]/(-\lambda_2^{(0)})\}\, e^{\lambda_2^{(0)}t} + \\ &+ [k_1/(\lambda_1^{(0)} - \lambda_2^{(0)})]\,[k_2 - (k_2 + \lambda_1^{(0)})\,z]/(-\lambda_1^{(0)})\, e^{\lambda_1^{(0)}t} \end{aligned} \tag{10}$$

where

$$\lambda_{1,2}^{(0)} = -(k_{-1} + k_1 + k_2)\left(1 \pm \sqrt{1 - 4k_1k_2/(k_{-1} + k_1 + k_2)^2}\right)/2$$

The standard deviations are rigorously zero at t = 0.

They assume their maxima when $\mathrm{E}(\xi) = 1/2$ or $\mathrm{E}(\zeta) = 1/2$, as they can be given by equation (11)

$$\mathrm{D}(\xi) = [\mathrm{E}(\xi) - \mathrm{E}^2(\xi)]^{1/2}, \quad \mathrm{D}(\zeta) = [\mathrm{E}(\zeta) - \mathrm{E}^2(\zeta)]^{1/2} \tag{11}$$

(Note that ξ and ζ can assume only 0 and 1.)

The standard deviations tend to zero when $t \to \infty$, but the coefficient of variation $\mathrm{D}(\zeta)/\mathrm{E}(\zeta)$ is growing to infinity.

It can be seen that, depending on the actual values of the rate constants k_1, k_{-1}, and k_2, the differences between the stochastic means and the deterministic solution may assume relatively large values for the substrate, even 20–30 per

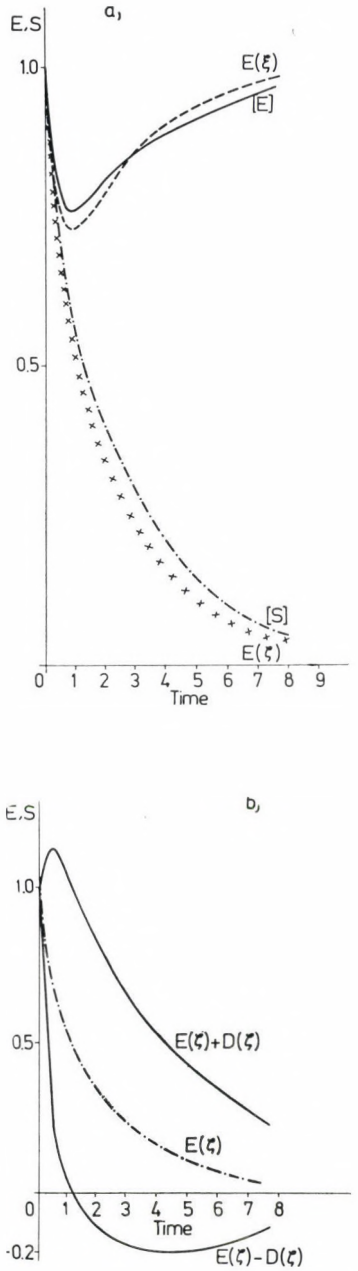

Fig. 1. Time course of the Michaelis-Menten reaction. $k_1 = 1$, $k_{-1} = 1$, $k_2 = 1$. Solutions to equation (8) ([E], [S]) and stochastic expected values [E(ξ), E(ζ)] are compared (a). The stochastic expected value E(ζ) is plotted together with its variance (b).

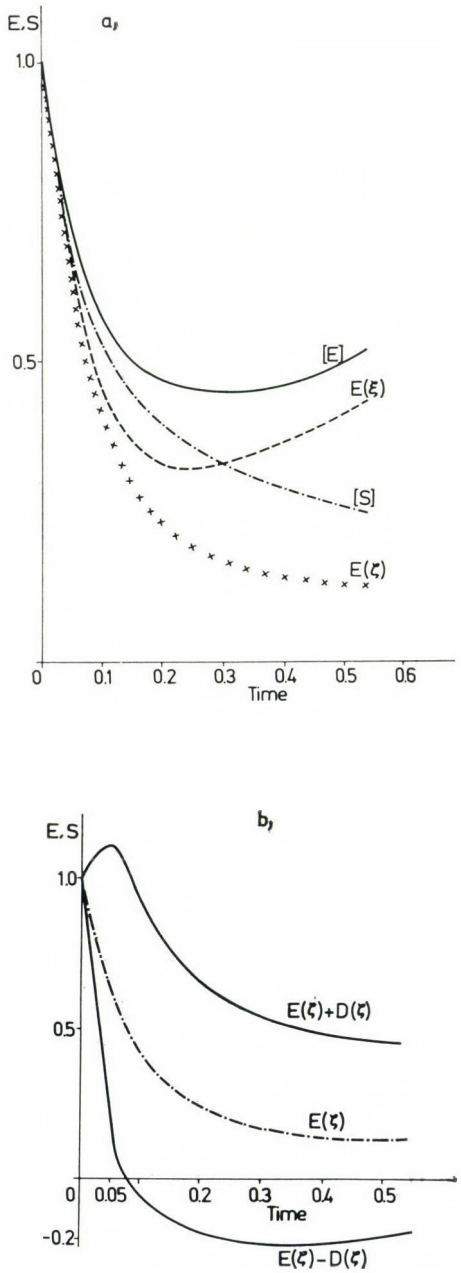

Fig. 2. Time course of the Michaelis-Menten reaction. $k_1 = 10$, $k_{-1} = 2$, $k_2 = 1$. Solutions to equation (8) ([E], [S]) and stochastic expected values [E(ξ), E(ζ)] are compared (a). The stochastic expected value E (ζ) is plotted together with its variance (b).

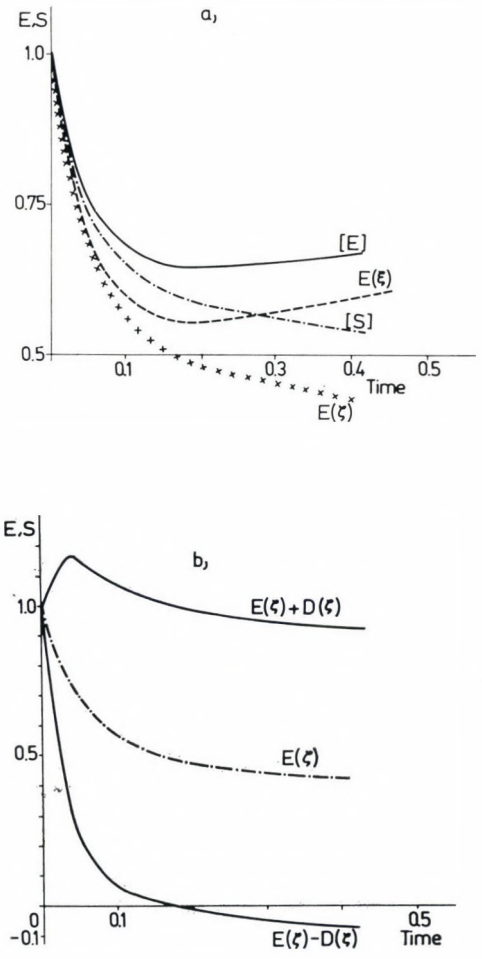

Fig. 3. Time course of the Michaelis-Menten reaction. $k_1 = k_{-1} = 10$, $k_2 = 1$. Solutions to equation (8) ([E], [S]) and stochastic expected values [E(ξ), E(ζ)] are compared (a). The stochastic expexted value E(ζ) is plotted together with its variance (b).

cent of the deterministic functions, which, as a rule, always run higher. This difference can by no means be neglected *a priori*.

Fig. 4.a shows for comparison the function $\tilde{S}(t)$ obtained by assuming a steady state throughout the reaction. Not much to our astonishment at the beginning of the reaction it approximates the deterministic solution to a lesser extent than the stochastic mean. Namely, the latter ones are exactly the same as $t \to 0$ (or $t \to \infty$) due to the initial conditions (4).

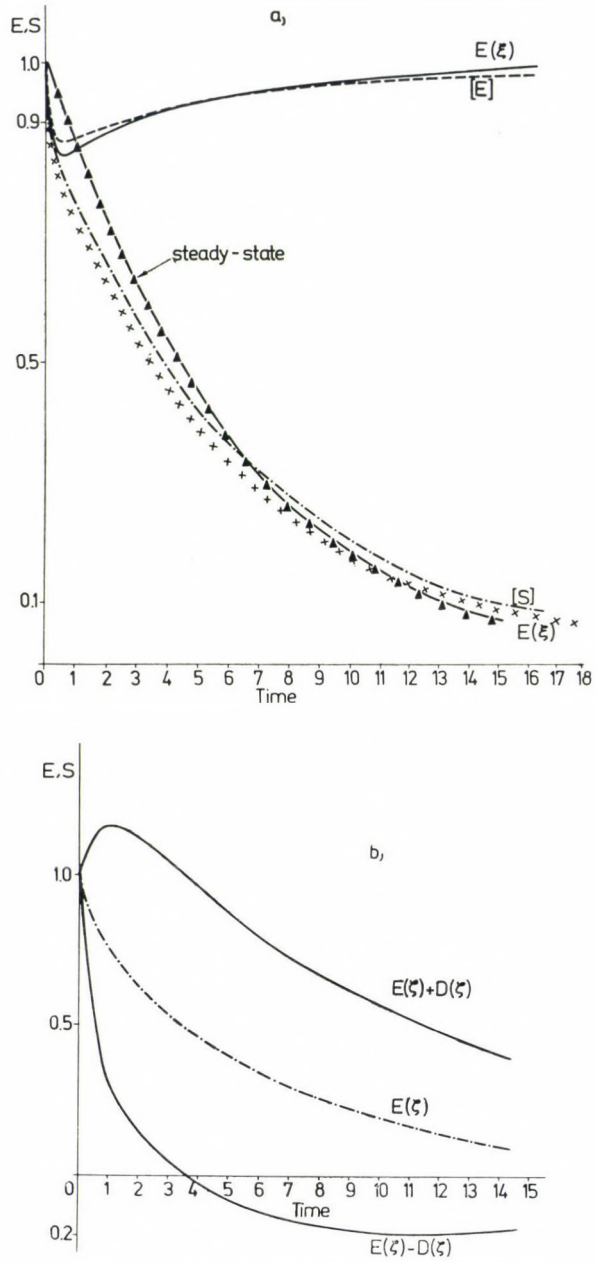

Fig. 4. Time course of the Michaelis-Menten reaction $k_1 = 2$ $k_{-1} = 10$ $k_2 = 1$. Solutions to equation (8) ([E], [S]) and stochastic expected values [E(ξ), E(ζ)] are compared (a). The stochastic expected value E(ζ) is plotted together with its variance (b). The steady state approximation to the function [S] (t) is also given (a).

The reversible reaction

The basic assumptions on which a stochastic model is based for the reaction

$$E + S \leftrightharpoons C \leftrightharpoons E + P \tag{II}$$

are identical with those itemized in section 2.a, except that product molecules are allowed to interact with the enzyme (cf, assumption 5). Correspondingly, we have to add a reaction to Table 1 of infinitesimal transition probabilities:

$$E + P \xrightarrow{k_{-2}} C$$

This reaction means a transition $(e, s) \to (e-1, s)$ with an infinitesimal transition probability $k_{-2}e\ (S_0 - s - 1 + e)\ \Delta t$.

The absolute probabilities can be determined in the same way as for reaction (I). Here only equilibrium will be considered in detail.

The Markov process defined for reaction (II) is irreducible. Its state space is finite and so it has a unique limit distribution (Rényi, 1954). So the problem of investigating the equilibrium is well set in this sense.

Introducing the following notation

$$F_e(z) = \lim_{t \to \infty} G_e(z, t)$$

we have the following partial differential equations

$$\begin{aligned}(k_{-1} - k_{-2}z)\frac{dF_1(z)}{dz} &= -k_{-2}S_0F_1(z) + (k_{-1} + k_2)F_0(z)\\ (k_{-2} - k_1)z\frac{dF_1(z)}{dz} &= k_{-2}S_0F_1(z) - (k_{-1}z + k_2)F_0(z)\end{aligned} \tag{12}$$

and the condition

$$F_0(1) + F_1(1) = 1 \tag{13}$$

whose solutions are (Arányi, 1976):

$$\begin{aligned}F_0(z) &= [k_1k_{-2}S_0(k_{-1}k_{-2} + k_1k_2 + k_1k_{-2}S_0)]\,[(k_{-1}k_{-2}z + k_1k_2)/(k_{-1}k_{-2} + k_1k_2)]^{S_0 - 1}\\ F_1(z) &= [(k_{-1}k_{-2} + k_1k_2)/(k_{-1}k_{-2} + k_1k_2 + k_1k_{-2}S_0)] \times\\ &\quad \times [(k_{-1}k_{-2}z + k_1k_2)/(k_{-1}k_{-2} + k_1k_2)]^{S_0}\end{aligned} \tag{14}$$

Solution to the general problem when $k_1 = k_{-2}$ was given by Darvey and Staff (1967), and, without restriction by Staff (1970). Again, however, the case of few molecules has not been discussed in detail. The absolute probability functions can be obtained from (14) using equation (6) after taking the limit $t \to \infty$ at both

sides. For comparison with the deterministic model we give an expression for the stochastic mean

$$E(\zeta(\infty)) = \frac{k_1 k_{-1} k_{-2}^2 S_0^2}{(k_{-1}k_{-2}+k_1k_2)(k_{-1}k_{-2}+k_1k_2+k_1k_{-2}S_0)} + \\ + \frac{(k_{-1}k_{-2} + k_1k_2 - k_1k_{-2})\, k_{-1}k_2 S_0}{(k_{-1}k_{-2}+k_1k_2)(k_{-1}k_{-2} + k_1k_2 + k_1k_{-2}S_0)} \tag{15}$$

Its deterministic counterpart from the condition $\frac{d[E]}{dt} = \frac{d[S]}{dt} = 0$ is

$$[S](\infty) = \frac{k_{-1}}{2k_1(k_{-1}k_{-2} + k_1k_2)} \{[(S_0 - 1)k_1k_{-2} - k_1k_2 - k_{-1}k_{-2}] + \\ + [(k_1k_{-2}(S_0 + 1) + k_1k_2 + k_{-1}k_{-2})^2 - 4k_1^2k_{-2}^2S_0]\} \tag{16}$$

When S_0 assumes large values, both $E(\zeta(\infty))$ and $[S](\infty)$ approach $S_0 = \frac{k_{-1}k_{-2}}{k_{-1}k_{-2}+k_1k_2}$ i.e. the difference between the stochastic and deterministic equilibria vanishes. In Table 2 numerical values are given for $E(\zeta(\infty))$ and $[S](\infty)$ at different k sets and S_0 values.

Table 2

Comparison of deterministic substrate molecule numbers with stochastic means at equilibrium

S_0	k_{-2}	k_{-1}	k_1	k_2	$E(\xi(\infty))$	$[S](\infty)$	$\frac{[S](\infty) - E(\xi(\infty))}{[S](\infty)}$
1	2	1	1	1	0.400	0.457	0.125
1	10	1	1	1	0.476	0.577	0.175
1	100	1	1	1	0.498	0.614	0.189
1	1000	1	1	1	0.500	0.618	0.191
3	10	1	1	1	2 062	2.110	0.023
10	10	1	1	1	8.272	8.280	0.001
1	100	1	120	1	0.008	0.058	0.862
1	15	1	10	1	0.086	0.200	0.570
3	15	1	10	1	1.232	1.245	0.010
10	15	1	10	1	5.410	5.411	0.000
1	1	1	1000	1	0.000500	0.000618	0.191
1	1	1	10	1	0.048	0.058	0.172
3	1	1	10	1	0.206	0.211	0.024
10	1	1	10	1	0.827	0.828	0.001
1	2	10	1	10	0.625	0.627	0.003
3	2	10	1	10	1.889	1.894	0.003
10	2	10	1	10	6.400	6.406	0.001

Conclusions

What inferences can be drawn for real systems from a stochastic model which is based upon the assumption that only one enzyme molecule is present in a reaction vessel?

As the intricate reaction pathways in the living cell are highly compartmentalized for the sake of regulation (or for other reasons) (Friedrich, 1974), it may easily happen that only a single enzyme molecule will be active in one compartment.

We saw that in this case stochastic and deterministic descriptions give significantly different results when means are compared with the solutions of equations (8). Of course, the biochemist observes the average of thousands of millions of such compartments. However, if they function independently, stochastic means sum up, and the difference between the results obtained from the two models does not disappear, although the coefficients of variation tend to zero. That is to say the expected value is of crucial importance from an experimental point of view. If the enzyme catalyzes a process vital for the cell, the consequences of stochastic uncertainty, i.e. variance, would also play an important role.

Indirec ly, one can conclude that systems which contain more than one, but not too many enzyme molecules may display a behaviour that reminds one of the one-enzyme-molecule case rather than that of infinitely many molecules.

The mathematical means introduced here and developed elsewhere (Arányi, 1976) allow us to investigate these systems directly. The extension of systems (1) and (2) to cases when $P(e>1)>0$ is straightforward.

Since in the living cells there exist molecules of vital importance other than enzymes of Michaelis-Menten behaviour (mainly nucleic acids) that are definitively known to be present in a few copies only, it seems to be worth-while to examine time courses or equilibria of their possible reactions in a similar way.

Finally, from a computational point of view we can recommend the use of stochastic mean functions when the situation is clearly closer to deterministic, but the exact solution of equation (8) cannot be obtained, and steady state approximation does not seem to apply.

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References

Arányi, P. (1976) Univ. thesis, Eötvös Loránd University, Budapest.
Bartholomay, A. F. (1962) Biochemistry *1* 223—230
Darvey, I. O., Staff, P. J. (1967) J. Theor. Biol. *14* 157—172
Delbrück, M. (1940) J. Chem Phys. *8* 120—124
Friedrich, P. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. *9* 159—173
Heyde, C. C., Heyde, E. (1969) J. Theor. Biol. *25* 159—172

Heyde, C. C., Heyde, E. (1971) J. Theor. Biol. *30* 395–404
Jachimowski, C. J., McQuerrie, D. A., Russell, M. E. (1964) Biochemistry *3* 1732–1736
Rényi, A. (1954) Probability theory (in Hungarian), Tankönyvkiadó, Budapest, pp. 625–626
Smeach, S. C., Gold, H. J. (1975a) J. Theor. Biol. *51* 59–78
Smeach, S. C., Gold, H. J. (1975b) J. Theor. Biol. *51* 79–96
Smeach, S. C., Smith, W. (1973) J. Theor. Biol. *42* 157–167
Staff, P. J. (1970) J. Theor. Biol. *27* 221–232
Stuart, R. N., Branscombe, E. W. (1971) J. Theor. Biol. *31* 313–329

Appendix

Proof of ergodicity of finite Markov process with an absorbing state, accessible from any other state. Let state 0 be absorbing.

Then $P_{0j}(t)$ transition probabilities are 0 if $j \neq 0$ or 1 if $j = 0$. Examine separately $\lim_{t\to\infty} P_{i0}(t)$ $(i \neq 0)$ and $\lim_{t\to\infty} P_{ij}(t)$ $(i, j \neq 0)$. As state 0 is accessible from any state, $\min_{l\neq 0} P_{l0}(t_0) = q > 0$ for $t_0 > 0$. Now, if $t = nt_0 + h$ $(0 < h < t_0)$ it is easy to realize that

$$P_{ij}(t) = P_{ij}(nt_0 + h) \leq \sum_{k\neq 0} P_{ik}(nt_0)\, P_{kj}(h) \leq \sum_{k\neq 0} P_{ik}(nt_0) =$$

$$= \sum_{k\neq 0} \sum_{l\neq 0} P_{il}[(n-1)\,t_0]\, P_{lk}(t_0) \leq (1-q) \sum_{l\neq 0} P_{il}[(n-1)\,t_0] \leq (1-q)^n$$

As $n \to \infty$ with $t \to \infty$,

$$\lim_{t\to\infty} P_{ij}(t) = 0 \quad (i, j \neq 0)\,,$$

For proving $\lim P_{i0}(t) = 1$ consider that $1 - P_{i0}(t) = \sum_{k\neq 0}^{t\to\infty} P_{ik}(t)$. This sum tends to zero as $t \to \infty$, as it consists of a finite number of terms.

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Regulation of Phosphorylase-Phosphatase from Skeletal Muscle by Phosphorylation of a Regulator Protein

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The inhibitory effect of a heat-stable regulator protein from skeletal muscle on the activity of phosphorylase-phosphatase (EC 3.1.3.17) was studied. The regulator protein was shown to be both phosphorylated and dephosphorylated in vivo as well as in vitro. The incorporation of phosphate into the regulator protein increased, while dephosphorylation decreased the ability of the protein to inhibit phosphatase activity. Our results suggest that the reversible phosphorylation of the regulator protein plays an essential role in the regulation of phosphorylase-phosphatase activity.

Introduction

Covalent modification of enzymes has been found to be one of the basic mechanisms of the regulation of enzyme activities. Phosphorylase (EC. 2.4.1.1), an enzyme which mobilizes glycogen, is activated trough incorporation of phosphate, while its inactivation is brought about by dephosphorylation. Phosphorylation and dephosphorylation are catalyzed by phosphorylase-kinase (EC 2.7.1.38) and phosphorylase-phosphatase (EC 3.1.3.17), respectively.

Phosphorylase-kinase itself is activated by Ca^{++} ions and a cAMP dependent protein kinase. The activation process has been studied in detail. Little is known, however, about the factors affecting the activity of phosporylase-phosphatase. The regulation of this activity is of great importance because this enzyme influences the "life time" of phosphorylase *a* and in this way, the extent of glycogen breakdown.

It has been shown that the activity of phosphorylase-phosphatase in the protein-glycogen complex is transiently inhibited when the phosphorylase $b \rightarrow a$

Abbreviations: AMP, adenosine-5'-monophosphate; ATP, adenosine-5':triphosphate; ATP-γ-S, adenosine-5'-0-(3-thioltriphosphate); cAMP, adenosine-3':5'-monophosphate; EDTA, ethylenediaminotetraacetic acid; EGTA, ethyleneglycol-bis(aminoethylether)-N,N'-tetraacetate; P_i, inorganic phosphate; SDS, sodiumdodecylsulfate; Tris, tris(hydroxymethyl)-aminomethane, HCl.

conversion takes place in the complex (Meyer et al., 1970); Heilmeyer et al., 1970; Hascke et al., 1970, 1972).

According to Bot et al. (1975b, 1975a) the activity of phosphorylase-phosphatase is inhibited by phosphorylase-kinase. Phosphorylation of phosphorylase-kinase substantially increases this inhibition (Gergely et al., 1976). It could be shown by frontal analysis gel filtration that the inhibition is due to protein-protein interaction (Gergely et al., 1974, 1975).

Besides phosphorylase-kinase, however, a heat-stable regulator protein also plays a role in the regulation of the activity of phosphorylase-phosphatase. From liver or skeletal muscle a heat-stable protein fraction, significantly inhibiting the activity of phosphorylase-phosphatase, could be prepared (Brandt et al., 1974, 1975a, 1975b).

In this paper, we shall demonstrate that the inhibitory effect of this protein fraction on the activity of phosphorylase-phosphatase depends on the phosphorylation-dephosphorylation reactions in vivo as well as in vitro.

Materials and methods

Preparation of enzymes and determination of enzyme activities

Phosphorylase *b* was prepared according to Fischer and Krebs (1962) and was three times recrystallized from 0.04 M glycerophosphate + 0.01 M mercaptoethanol (pH 6.8).

Phosphorylase *a* was produced from phosphorylase *b* by phosphorylase-kinase in the presence of 0.012 M Mg^{++} and 0.003 M ATP (Fischer, Krebs, 1962). The enzyme was recrystallized three times, then nucleotides were removed by dialysis against 0.04 M citrate + 0.4 M imidazole buffer (pH 6.8) as described by Griffiths et al. (1974). The A_{260}:A_{280} ratio of the enzyme obtained in this way was in good agreement with the data in the literature. The preparation had a specific activity of 58 units/mg protein when measured as described below.

The activity of phosphorylase *a* was measured by the method of Illingworth and Cori (1953) in the direction of glycogen synthesis. P_i liberated from glucose-1-phosphate present at a concentration of 0 016 M was determined according to Taussky and Shorr (1953). One unit was defined as the amount of the enzyme capable of liberating μmole of P_i in 1 minute under these conditions.

Phosphorylase-phosphatase was prepared from rabbit skeletal muscle as described by Brandt et al. (1974).

The activity of phosphorylase-phosphatase was determined according to Bot et al. (1975a). 25 units of phosphorylase *a* were incubated at 30°C in the presence of phosphorylase-phosphatase in a buffer containing 0.05 M imidazole + 0.005 M EDTA + 0.005 M dithiotreitol (pH 7.4). The conditions of the incubation were chosen such as to allow the tranfsormation of not more than half the amount of phosphorylase *a* into inactive phosphorylase *b*. The reaction was stopped by the

addition of NaF, then the activity of the remaining phosphorylase *a* was measured. One unit of phosphorylase-phosphatase was defined as the amount inactivating 0.2 mg of phosphorylase *a* in 1 minute (Killilea et al., 1976a).

The activity of phosphorylase-kinase was measured according to Krebs et al. (1964) at pH 6.8 and 8.2. The ratio of activities measured at these two pH values is characteristic of the degree of activation of the enzyme (De Lange et al., 1968). The activity measured at pH 6.8 was expressed as the percentage of that measured at pH 8.2.

Protein concentration was determined by the method of Hartree (1972).

Preparation of the protein regulating phosphorylase-phosphatase and the measurement of its inhibitory effect

The regulator protein was prepared according to Brandt et al. (1975a) with slight modifications. Rabbit skeletal muscle extract was prepared with 3 volumes of 0.05 M imidazole + 0.005 M EDTA + 0.01 M mercaptoethanol (pH 7.4.) The homogenate was centrifuged at 10 000 *g* for 20 minutes at 0°C. The supernatant was kept at 90°C for 5 minutes. The precipitated proteins were homogenized in a glass homogenizer for one minute and removed by centrifugation. Ammonium sulfate was added to 75% saturation, to the supernatant containing the regulator protein.

After 15 minutes the precipitated proteins were collected by centrifugation (10 000 g, 0°C, for 20 minutes). The pellet was dissolved in approximately three volumes of 0.05 M imidazole + 0.005 M EDTA +0.005 M dithiotreitol buffer (pH 7.4), and dialyzed against the same solution to remove ammonium sulfate.

The inhibitory effect of the regulator protein on phosphatase was determined by measuring phosphatase activity, i.e. by incubating phosphatase in the presence and absence of the regulator protein. Five to fifty micrograms of the regulator protein were incubated together with 25 units of phosphorylase *a* in a reaction mixture containing 0.05 M imidazole +0.005 M EDTA + 0.0005 M dithiotreitol (pH 7.4) + 0.02 M coffein + 0.1% glycogen. The reaction was carried out at 30 °C; it was started by the addition of the phosphatase and stopped by adding NaF to the mixture. The activity of the remaining phosphorylase *a* was measured. The capability of the regulator protein to inhibit phosphatase was expressed as the amount in units of phosphorylase-phosphatase inactivated by 1 mg of the regulator protein.

Phosphorylation of the proteins of skeletal muscle extracts

Freshly ground rabbit skeletal muscle was extracted by two volumes of 0.001 M EDTA for 10 minutes at 0 °C, the pH beeing kept at 7.0 by continuous addition of 2 M Tris solution. The extract was centrifuged at 5000 *g* for 30 minutes at 0 °C. The supernatant was filtered through glass-wool.

The proteins in the supernatant were phosphorylated in a mixture containing 0.025 M Tris + 0.025 M glycerophosphate buffer (pH 8.2) and 0.003 M

ATP + 0.012 M Mg^{++} + 10^{-5} M cAMP at 30 °C. In some experiments ATP-γ-S or [γ^{32}P] −ATP were used instead of ATP.

The reaction was started by adding cAMP. At different time intervals samples were taken and the phosphorylation process was stopped by the addition of a solution containing 0.1 M NaF + 0.02 M EDTA (pH 6.8). From one aliquot the amount of phosphorylase *a* formed and that of activated phosphorylase-kinase were determined. From another part of the sample, regulator protein was prepared by heat treatment and its ability to inhibit phosphatase was measured as described above.

Results

Our first aim was to investigate changes in the inhibitory action of the heat-stable regulator protein in connection with the phosphorylation-dephosphorylation reactions in the muscle. Phosphorylation of proteins in rat skeletal muscle was induced by s.c. injection of epinephrine. The time-course of phosphorylation was followed by measuring the activity of phosphorylase *a*. From the samples taken at different time intervals regulator protein was also prepared and its ability to inhibit phosphatase was determined.

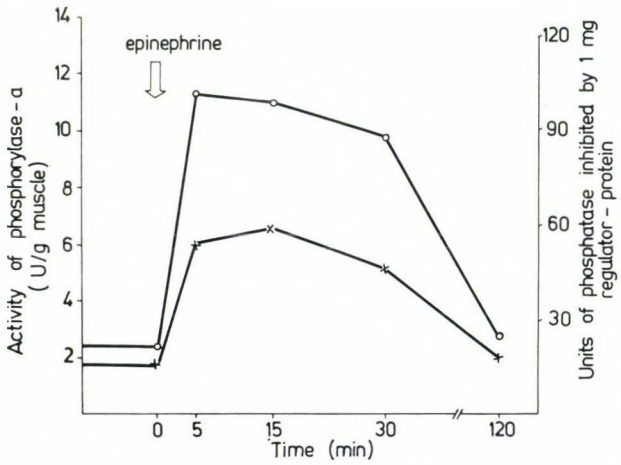

Fig. 1. Effect of epinephrine, on the activity of phosphorylase *a* from rat muscle and on the ability of the regulator protein to inhibit phosphatase. Hind legs of rats narcotized by Inactine were skinned. After 20 min sleep 400 μg/kg body weight of epinephrine were injected subcutaneously. After given time intervals ranging from 0 to 120 min, samples were taken from the muscle with Wollenberger forceps cooled in liquid air. The samples freezed in liquid air were pulverized, then 200 mg of muscle were homogenized in 5 ml of a buffer containing 0.1 M NaF+0.08 M glycerophosphate +0.005 M EGTA + 0.01 M mercaptoethanol (pH 6.8) at 0°. The homogenate was centrifuged at 5000 g for 20 min at 0° and the phosphorylase *a* activity of the supernatant was measured (○——○). From another part of the frozen muscle sample regulator protein was prepared and its ability to inhibit phosphatase was measured (×——×)

Figure 1. shows that the activity of phosphorylase *a* significantly increases on the addition of epinephrine concomitantly, the ability of the regulator protein to inhibit phosphatase also increases. The activity of phosphorylase *a* returns after 120 minutes to a value characteristic of resting muscle. The inhibitory activity of the regulator protein decreases in a parallel way.

The reversible increase in the inhibitory activity of the regulator protein as a consequence of phosphorylation was observed also in vitro.

Phosphorylation-dephosphorylation processes taking place in rabbit skeletal muscle extracts are shown in Fig. 2. Phosphorylation was carried out in the presence of cAMP, ATP and Mg^{++}. It can be seen that the activity of phosphorylase-kinase is greatly increased by phosphorylation. The activation of kinase is followed by the formation of phosphorylase *a*. After the increase in the activities of these two enzymes, first the kinase, then the phosphorylase are inactivated. This inactivation is a result of dephosphorylation which points to the action of phosphoprotein phosphatase(s).

Regulator protein prepared prior to the activation of the enzymes only slightly inhibits the activity of purified phosphorylase-phosphatase. In the course of phosphorylation, however, the inhibitory activity of the regulator protein significantly increases. Parallel with the decrease of the activity of phosphorylase-kinase and phosphorylase *a* (as a consequence of dephosphorylation), the inhibitory activity of the regulator protein also decreases.

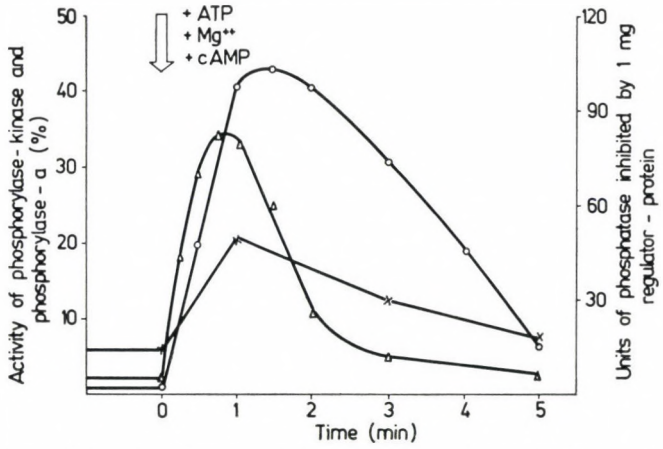

Fig. 2. Variation of phosphorylase *a* and phosphorylase-kinase activities and of the ability of the regulator protein to inhibit phosphatase in skeletal muscle extracts in vitro. Phosphorylation of proteins in skeletal muscle extracts was carried out in the presence of 0.03 M ATP + + 0.012 M Mg^{++} + 10^{-5} M cAMP as described in Materials and methods. Activities of the phosphorylase *a* formed and of phosphorylase-kinase were measured, regulator protein was prepared and its inhibitory activity determined as described in Materials and methods. △——△, phosphorylase-kinase activity at pH 6.8 expressed as the percentage of the activity measured at pH 8.2; ○——○, activity pf phosphorylase *a* expressed as the percentage of total phosphorylase activity measured in the presence of 1 mM AMP; ×——×, ability of regulator protein to inhibit phosphorylase-phosphatase

In order to demonstrate that the change in the activity of the regulator protein is due to its phosphorylation, the reaction was repeated using ATP-γ-S instead of ATP. In the presence of ATP-γ-S, thiophosphate groups are incorporated into the serine residues of proteins. Phosphatase is not able to inactivate thiophosphorylated proteins since it cannot remove the thiophosphate groups from the enzymes (Eckstein, 1966; Gratecos, Fischer, 1974; Gergely et. al., 1976).

Figure 3 shows the effect of thiophosphorylation on muscle extracts. It can be seen that, due to thiophosphorylation, phosphorylase-kinase is activated first and remains in this state. The incorporation of thiophosphate group, inhibiting the action of phosphatases, stabilizes the phosphorylase-kinase in an activated state. On thiophosphorylation of phosphorylase *b*, active thiophosphorylase is formed, the activity of which does not decrease upon further incubation. It is apparent that thiophosphorylation results in a tenfold increase in the inhibitory activity of the regulator protein. In contrast to normal phosphorylation (see Fig. 2), the increase in inhibitory activity brought about by thiophosphorylation does not diminish during further incubation.

According to these results, in the course of phosphorylation in muscle extracts not only phosphorylase-kinase and phosphorylase but the regulator protein itself is also phosphorylated. Phosphorylated regulator protein inhibits dephosphorylation of phosphorylase *a* significantly better than the non-phos-

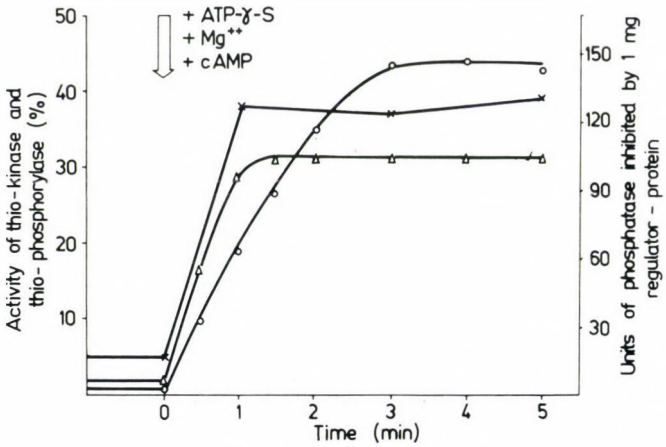

Fig. 3. Effect of thiophosphorylation on phosphorylase-kinase and phosphorylase activities and on the inhibitory activity of the regulator protein in muscle extracts. Phosphorylation of proteins in skeletal muscle extracts in the presence of 0.003 M ATP-γ-S + 0.012 M Mg^{++} + $+10^{-5}$M cAMP, measurement of the activities of thiophosphorylase and thio-kinase, preparation of the regulator protein and determination of its ability to inhibit phosphatase were carried out as described in Materials and methods. △——△ , thio-kinase activity at pH 6.8; ○——○ , thiophosphorylase activity in the absence of AMP expressed as the percentage of activity measured in the presence of 1 mM AMP; ×——× , inhibitory activity of regulator protein

phorylated form. However, the phosphorylated regulator protein itself is also dephosphorylated by phosphatases, which results in a substantial decrease of its inhibitory action. If the regulator protein is thiophosphorylated, dephosphorylation does not occur; thus the increased inhibitory activity remains unchanged.

In order to prove that the increase in the activity of the regulator protein is a consequence of its phosphorylation, the reaction was carried out by using [γ^{32}P]-ATP and the incorporation of ^{32}P into the regulator protein was studied.

Rabbit skeletal muscle extracts were phosphorylated in the presence of [γ^{32}P]-ATP, Mg^{++} and cAMP. Regulator protein, prepared from the extracts as described in Materials and methods, was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 4).

In this system the heat-stable regulator protein appears as three separate bands of different molecular weights. Radioactive phosphate is incorporated into the fraction with a molecular weight of 29 000 daltons. The other bands are not labelled.

We could also show that ^{32}P-labelled regulator protein is dephosphorylated in the presence of phosphorylase-phosphatase (Fig. 5).

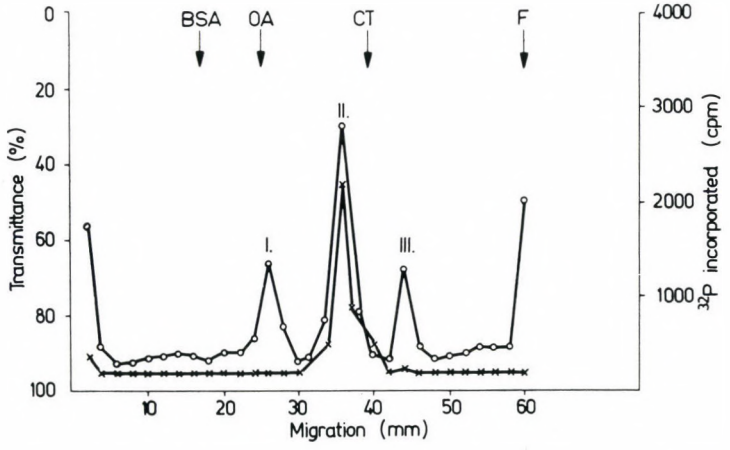

Fig. 4. SDS gel electrophoretogram of regulator protein phosphorylated by [$\gamma-^{32}$P] -ATP. Electrophoresis of the regulator protein was carried out by the method of Weber and Osborn (1969) in gels containing 7% acrylamide, 0.3% methylenebisacrylamide and 0.1% SDS. Protein content (transmittance) was determined in a densitometer at 550 nm. Gels were cut in to 1 mm slices, dissolved in 30% H_2O_2+0.2% NH_3 at 50° and the radioactivity of the samples was measured in a Packard Tricarb scintillation spectrometer. The arrows indicate the mobilities of proteins of known molecular weights: BSA (bovine serum albumin, Mw. 68 000), OA (ovalbumin, Mw. 45 000), CT (chymotrypsinogen, Mw. 25 000), F (bromophenolbue marker dye). Proteins were stained with Coomassie Brillant Blue R-250 dye. Molecular weights of the individual proteins: I, 40 000; II, 29 000; III, 20 000 daltons. ○——○ , transmittance (%, at 550 nm); ×——× , ^{32}P content (cpm)

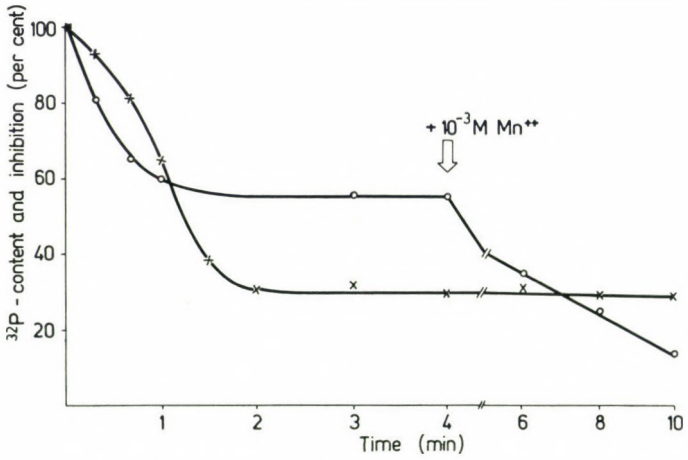

Fig. 5. Dephosphorylation of regulator protein labelled with ^{32}P in the presence of phosphorylase-phosphatase. Dephosphorylation of regulator protein (1.05 mg) labelled with ^{32}P was carried out in a buffer containing 0.05 M imidazole + 0.005 M EDTA + 0.0005 M dithiotreitol (pH 7.4) at 30°. The reaction was started by the addition of 0.2 mg of phosphorylase-phosphatase. O——O , decrease in the ^{32}P content of the regulator protein measured according to Gergely et al. (1976); ×——× , inhibitory activity of the regulator protein measured as described in Materials and methods

It is apparent from Fig. 5 that 40 – 45% of the ^{32}P content of the phosphorylated regulator protein is removed by phosphorylase-phosphatase. Parallel with this the inhibitory activity of the regulator protein shows a substantial decrease, to 30% of the original activity. The remaining ^{32}P content about 55% can be removed by phosphorylase-phosphatase only in the presence of Mn^{++}. The removal of this residual ^{32}P content, however, does not lead to a further decrease of the inhibitory activity of the regulator protein.

Discussion

Earlier data in the literature have suggested that the activity of phosphorylase-phosphatase is greatly influenced by phosphorylation processes. In crude extracts of skeletal muscle and adrenal cortex, a decrease in phosphorylase-phosphatase activity has been observed as an effect of ATP, Mg^{++} and cAMP (Merlevede, Riley, 1966; Chelala, Torres, 1970). Recently it has been shown that phosphorylase-phosphatase prepared from rabbit skeletal muscle is inactivated when incubated in the presence of cAMP-dependent protein kinase, cAMP, Mg^{++} and ATP (Huang, Glinsmann, 1975).

Up to the present the mechanism of the decrease of phosphorylase-phosphatase activity was not clear. We have found that the target of the phosphorylation processes is not phosphorylase-phosphatase itself but a heat-stable regulator protein, the phosphorylation of which influences the activity of phosphatase. The phosphorylation of this regulator protein by a cAMP-dependent protein kinase greatly enhances its ability to inhibit phosphatase. In this way the activity of phosphorylase-phosphatase is substantially reduced and this reduction of activity slows down the inactivation of phosphorylase *a*, thus lengthening the life-time of the enzyme. The ability of the regulator protein to inhibit phosphatase is decreased when it is dephosphorylated. Phosphorylase-phosphatase released from inhibition quickly inactivates phosphorylase *a* and this stops the breakdown of glycogen.

It is still unknown whether the heat-stable regulator protein exists as a separate protein in the cytoplasm or it is bound to (or possibly a subunit of) an enzyme participating in the regulation of glycogen breakdown. According to Brandt et al. (1975a) and Killilea et al. (1976b), this protein is a part of phosphorylase-phosphatase as its regulatory subunit. As it was mentioned in the introduction, also phosphorylase-kinase plays a significant role in the regulation of phosphorylase-phosphatase activity (Bot et al., 1975a, 1975b), and its phosphorylation – like that of the regulator protein – results in an increasing ability to inhibit phosphatases (Gergely et al., 1976). Unpublished results obtained in our laboratory indicate that a heat-stable protein inhibiting phosphorylase-phosphatase can be prepared from phosphorylase-kinase, too. It is possible that the regulator protein regulating the activity of phosphatase originates from phosphorylase-kinase and is a subunit of this enzyme. This assumption is supported by an earlier observation in our laboratory, i.e. that the inhibitory effect of phosphorylase-kinase on phosphorylase-phosphatase is due to a strong protein-protein interaction between the two enzymes (Gergely et al., 1974, 1975).

References

Bot, G., Varsányi, M., Gergely, P. (1975a) FEBS Lett. *50* 351–354

Bot, Gy., Gergely, P., Varsányi M,. Vereb, Gy.(1975b) MTA Biol. Oszt. Közl. *18* 397–407

Brandt, H., Killilea, S. D., Lee, E. Y. C. (1974) Biochim. Biophys. Res. Commun. *61* 598–604

Brandt, H., Lee, E. Y. C., Killilea, S. D. (1975a) Biochim. Biophys. Res. Commun. *63* 950–956

Brandt, H., Capulong, L. Z., Lee, E. Y. C. (1975b) J. Biol. Chem. Vol. *250* 8038–8044

Chelala, C. A., Torres, H. N. (1970) Biochim. Biophys. Acta *198* 504–513

De Lange, R. I., Kemp, R. G., Riley, D. V., Cooper, R. A., Krebs, E. G. (1968) J. Biol. Chem. *243* 2200–2208

Eckstein, F. (1966) J. Amer. Chem. Soc. *88* 4292–4294

Fischer, E. H., Krebs, E. G. (1962) in Methods in Enzymology (Colowick, S. P., Kaplan N. O., eds), Vol. *5* pp. 369–373, Acad. Press., New York

Gergely, P., Vereb, Gy., Bot, G. (1974) Acta Biochim. et Biophys. Acad. Sci. Hung. *9* 223—226
Gergely, P., Vereb, Gy., Bot, G. (1975) Acta Biochim. et Biophys. Acad. Sci. Hung. *10* 153—159
Gergely, P., Vereb, Gy., Bot, G. (1976) Biochim. Biophys. Acta *429* 809—816
Gratecos, D., Fischer, E. H. (1974) Biochim. Biophys. Res. Commun. *58* 960—967
Griffiths, J. R., Price, N. C., Radda, G. K. (1974) Biochim. Biophys. Acta *358* 275—280
Hartree, E. F. (1972) Anal. Biochem. *48* 422—427
Haschke, R. H., Heilmeyer, L. M. G., Meyer, F., Fischer, E. H. (1970) J. Biol. Chem. *245* 6657—6663
Haschke, R. H., Grätz, K. W., Heilmeyer, L. M. G. (1972) J. Biol. Chem. *247* 5351—5356
Heilmeyer, L. M. G., Meyer, F., Haschke, R. H., Fischer, E. H. (1970) J. Biol. Chem. *245* 6649—6656
Huang, F. L., Glinsmann, W. H. (1975) Proc. Nat. Acad. Sci. USA Vol. *72* 3004—3008
Huang, F. L., Glinsmann, W. H. (1976) FEBS Lett. Vol. *62* 326—329
Illingworth, B., Cori, G. T. (1953) in Biochemical Preparations (Snell, E. E., ed.) Vol. *3*, pp. 1—9, John Wiley et Sons, Inc., New York
Killilea, S. D., Brandt, H., Lee, E. Y. C., Whelan, W. J. (1976a) J. Biol. Chem. *251* 2363—2368
Killilea, S. D., Brandt, H., Lee, E. Y. C. (1976b) Trends in Biochem. Sci. *1* 30—33
Krebs, E. G., Fischer, E. H. (1962) in Methods in Enzymology (Colowick, S. P., Kaplan, N. O., eds), Vol. *5* pp. 373—376, Acad. Press., New York
Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., Fischer, E. H. (1964) Biochemistry *3* 1022—1033
Merlevede, W., Riley, G. A. (1966) J. Biol. Chem. *241* 3517—3524
Meyer, F., Heilmeyer, L. M. G., Haschke, R. H., Fischer, E. H. (1970) J. Biol. Chem. *245* 6642—6648
Taussky, H. H., Shorr, E. (1953) J. Biol. Chem. *202* 675—685
Weber, K., Osborn, M. (1969) J. Biol. Chem. *224* 4406—4412

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Substrate Specificity of Acylase-I-Catalyzed Dipeptide Hydrolysis

(Short Communication)

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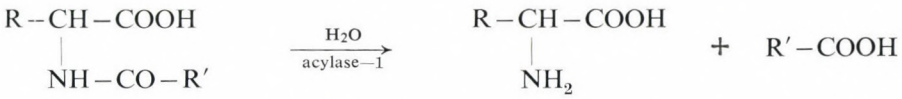
Acylase-I (aminoacylase, N-acylamino acid amidohydrolase, EC 3.5.1.14) catalyzing the hydrolysis of different N-acylamino acids (Greenstein and Winitz, 1961)



is one of the most important enzymes applied in preparative organic chemistry (Birnbaum et al., 1952). Earlier we studied the mechanism (Ötvös et al., 1971) and stereochemistry (Ötvös et al., 1975) of acylamino acid hydrolysis. In contrast to the abundant literature on acylase-I, only a few data are available concerning the hydrolysis of peptides (Rao et al., 1952; Kördel and Schneider, 1975). In our preceding paper (Ötvös et al., 1977) we have shown that the specificity of the acylase-I-catalyzed hydrolysis of dipeptides containing L-norvaline as the C-terminal amino acid is merely of a kinetic nature. Interpretation of the stereospecificity was based on a fixed conformation of the substrates in the ES-complex. For a detailed study of the specificity of peptide hydrolysis, the hydrolysis of dipeptides and substituted dipeptides containing systematically selected amino acids both in the C- and N-terminal moieties is now under investigation. In order to extend our conclusions drawn in our previous paper on the hydrolysis of L-norvaline derivatives (Ötvös et al., 1977), in this paper we report the hydrolysis of dipeptides containing glycine, L-alanine and L-norvaline on the C-terminal.

The substrates were synthesized by the phosphazo method according to Goldschmidt and Lautenschlager (1953). Physical data of the new compounds are summarized in Table 1.

All materials were purified to constant melting point and optical rotation. Purity was checked by elementary analysis, IR and NMR spectrometry as well as thin layer chromatography. Acylase-I was purchased from Sigma Chemical Company.

Abbreviations used: Nva, norvaline; Phg, C-phenylglycine.

Table 1

Physical and analytical properties of the substrates

$[\alpha]_D$ values were measured at 22 °C in H_2O using a 1 dm polarimeter tube

Compounds	$[\alpha]_D^{22}$ degree	conc. %	Calcd.			Found		
			N	C	H	N	C	H
			%			%		
L-Nva-Gly	−82.8	2	16.09	48.28	8.05	16.03	48.90	8.04
D-Nva-Gly	+85.7	2	16.09	48.28	8.05	15.75	47.98	8.03
L-Nva-L-Ala	+ 6.9	1	14.89	51.06	8.51	15.24	51.14	8.83
D-Nva-L-Ala	−70.8	1	14.89	51.06	8.51	14.52	50.58	8.64
L-Phg-L-Nva	−45.9*	2.1	11.20	62.40	7.20	10.95	61.96	8.00
D-Phg-L-Nva	−11.3*	2.1	11.20	62.40	7.20	11.05	61.85	7.90

* Optical rotation was measured in 0.15 M phosphate buffer.

The hydrolysis of dipeptides was carried out at pH 7.3 at 37 ± 0.1 °C in 0.15 M phosphate buffer. The rate of hydrolysis was measured by monitoring the loss of ultraviolet absorption of the peptide bond (228–235 nm) according to Schmitt and Siebert (1961). Changes in optical density were recorded with an UNICAM SP 8000 spectrophotometer. $K_{m(app)}$ and V_{max} values were determined from the Lineweaver-Burk plots.

The k_{cat} values were calculated by the use of data characteristic of acylase-I and published by Bruns and Schulze (1962). Inhibition constants (K_I) and the type of inhibition were determined by the method described by Dixon and Webb (1964). In each experiment N-acetyl-L-methionine was employed as a substrate in 5 different concentrations ($1 \times 10^{-3} - 1 \times 10^{-2}$ M). The concentration of the inhibitors varied between 1×10^{-2} and 8×10^{-2} M. Five inhibitor concentrations were used for the determination of K_I.

The kinetic constants are listed in Table 2.

Earlier (Ötvös et al., 1971) it was established that the decomposition of the ES complex was the rate-controlling step of the reaction and that $K_{m(app)}$ was equal to K_S. Accordingly, $K_{m(app)}$ is characteristic of the binding between the substrate and the enzyme. In a subsequent paper (Ötvös et al., 1977), interpretation of the specificity of C-terminal norvaline dipeptides was based on the assumption, that the substrate was fixed in the ES complex by the C-terminal carboxylate and alkyl side chain, on the one hand, and by the N-terminal amino group, on the other. Depending on its bulkiness the alkyl side chain in the N-acyl group hinders the reaction between the catalytic site of the enzyme and the carbonyl group of the substrate. Our recent results (Ötvös et al., 1977), with C-terminal norvaline peptides as well as glycine and alanine peptides also support this

Table 2

Kinetic constants of the acylase-I catalyzed hydrolysis of dipeptides

The initial rates were determined uv spectrophotometrically at pH 7.3 and 37 °C in 0.15 M phosphate buffer

Compounds	$K_{m(app)}$	K_I	k_{cat} sec^{-1}	k_{cat}/K $mM^{-1}sec^{-1}$
	mM			
(-Gly)				
Gly-	18.6		3.5	0.19
L-Ala-	8.0		2.1	0.26
D-Ala-	—	4.2	0	0
L-Val-	4.3		1.1	0.26
L-Nva-	3.6		2.3	0.65
D-Nva-	—	7.0	0	0
L-Phe-	6.2		0.34	0.05
(-L-Ala)				
Gly-	13.2		17.0	1.3
L-Ala-	5.4		15.8	2.9
D-Ala-	—	3.7	0	0
L-Val-	2.8		1.6	0.57
L-Nva-	3.4		10.6	3.1
(-L-Nva)				
Gly-	3.2		170	53
L-Ala-	2.2		155	70
D-Ala-	2.5		0.85	0.34
L-Val-	2.0		1.2	0.60
L-Nva-	2.7		102	38
D-Nva-	2.2		0.13	0.06
L-Phg-	1.4		0.40	0.28
D-Phg-	—	2.1	0	0

assumption, as can be seen by comparing the k_{cat} values in Table 2. The k_{cat} constants of valyl-, phenylglycyl- and phenylalanyl dipeptides unambiguously point to steric hindrance by N-terminal amino acid side chains.

An analysis of the $K_{m(app)}$ and K_I values allows the following conclusions:

a) The K_I values of the D−L compounds are nearly identical with the $K_{m(app)}$ constants of the corresponding L−L substrates. This equivalence indicates that the observed $K_{m(app)}$ value is a true binding constant (K_S). This supports our earlier findings (Ötvös et al., 1971).

b) The difference in the $K_{m(app)}$ values is not greater than two-fold in the individual substrate series except for glycyl-glycine and glycyl-L-alanine, which contain no hydrophobic side chains. This fact suggests that, with respect to specificity, the difference in the hydrophobic interactions of N-terminal amino acid side chains is of much less importance than the difference in their bulkiness.

c) The amino group plays the major role in the binding of the N-acyl moiety. According to Kördel and Schneider (1975) and our own studies on pH-dependence (to be published later), ionic interaction between the amino group of the substrate and the enzyme is unlikely. The amino group may be bound to the enzyme by hydrogen bonding.

d) The hydrophobic interaction of the C-terminal amino acid with the enzyme can be seen by comparing the $K_{m(app)}$ values of Gly-Gly, Gly-Ala and Gly-Nva. The $K_{m(app)}$ constant of the last substrate is six times as small as that of Gly-Gly, consequently, hydrophobic interaction between the side chain of the C-terminal amino acid and the enzyme is significant. This statement is in good agreement with the findings of Marshall et al. (1956) as well as those of Kördel and Schneider (1975) and our results (not yet published) with other N-acylamino acids.

e) Although steric hindrance of the reaction center by the N-acyl side chain is decisive in specificity as pointed out in *b)*, differences in the ratios of the individual $K_{m(app)}$ values (Ala-Gly/Ala-Nva = 3.6; Val-Gly/Val-Nva = 2.2, and Nva-Gly/Nva-Nva = 1.3) show that hydrophobic interaction of the N-terminal side chain must also be taken into account.

The configuration of the N-terminal amino acid has a decisive effect on the hydrolysis catalyzed by acylase-I. Dipeptides containing D-amino acids in the acyl moiety in general are not hydrolyzed. These compounds were tested as inhibitors (Table 2). D-Ala- and D-Nva-L-Nva show very slow hydrolysis but the reactivities of these diastereoisomers are by orders of magnitudes lower than those of L-Ala-L-Nva and L-Nva-L-Nva. This observation is in agreement with our earlier interpretations (Ötvös et al., 1975).

In conclusion, the results presented here suggest that the specificity of dipeptide hydrolysis catalyzed by acylase-I is mainly of a kinetic nature.

References

Birnbaum, S. M., Levintov, L., Kingsley, R. B., Greenstein, J. P. (1952) J. Biol. Chem. *194* 455—467

Bruns, F. H., Schulze, Ch. (1962) Biochem. Z. *336* 162—181

Dixon, M., Webb, E. C. (1964) Enzymes, Chap. 2. pp. 8—12. Acad. Press, N. Y.

Goldschmidt, S., Lautenschlager, H. (1953) Ann. *580* 68—79

Greenstein, J. P., Winitz, M. (1961) Chemistry of the amino acids pp. 1753—1767, Wiley and Sons Inc., New York

Kördel, W., Schneider, F. (1975) Hoppe-Seyler's Z. Physiol. Chem. *356* 915—920

Marshall, R., Birnbaum, S. M., Greenstein, J. P. (1956) J. Am. Chem. Soc. *78* 4636—4642

Ötvös, L., Moravcsik, E., Mády, Gy. (1971) Biochem. Biophys. Res. Commun. *44* 1056—1063

Ötvös, L., Moravcsik, E., Kraicsovits, F. (1975) Tetrahedron Lett. *29* 2485—2488

Ötvös, L., Moravcsik, E., Mády, Gy., Tüdős, H., Telegdi, J. (1977) Acta Chim. Hung. *94* 221—227

Rao, K. R., Birnbaum, S. M., Kingsley, R. B., Greenstein, J. P. (1952) J. Biol. Chem. *198* 507—523

Schmitt, A., Siebert, G. (1961) Biochem. Z. *334* 96—107

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Excitatory Effects of the Electrical Activity of Muscle on the Contiguous Nerve

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Experiments were performed on frog sciatic-gastrocnemius preparations in order to investigate the different conditions under which the excitation can be transmitted from muscle to nerve.

The results demonstrate that, on one hand, the proximal part of the m. gastrocnemius is of greater importance in the transmission of excitation than the distal one and, on the other hand, when the nerve is in contact with more than one muscle, the transmission of excitation is determined by the compound electrical activity of the muscles.

These results together with the findings of other authors support the hypothesis that the effect of the electrical muscle activity on the nerve in contact with the muscles could play an additional role in the mechanism of the excitatory processes of both the nerve and muscle.

Introduction

Taking Matteucci's experiments (1842) as a starting point, our results have demonstrated that the excitation can be transmitted through several nerve-muscle preparations forming in a chain (Biró, 1975) and the nerve-muscle preparations can produce a long-lasting excitation if arranged into special structures (Biró, 1977) and the transmission of excitation from muscle to nerve can take place under in situ circumstances, as well (Biró, 1975; Biró, Vu-Duy-Thinh, 1977). In connection with these results it has been emphasized that the excitation was not transmitted in every position of the nerves on the muscles, however, it was possible to find a favourable position for the transmission of excitation in most cases.

The aim of the present work was to investigate the circumstances which determine the transmission of excitation from muscle to nerve.

Methods

The experiments were performed on sciatic-gastrocnemius preparations of frogs *(Rana esculenta)*. In some of the experiments whole leg preparations were also used.

The excitation of the preparations was elicited with square-wave impulses given to the proximal part of the sciatic nerve and to the sciatic plexus in the cases of the nerve-muscle and the leg preparations, respectively. The parameters of the stimuli were 2 V in amplitude and 0.1 ms in duration except for one series of experiments in which the stimulus amplitude was changed. The pairs of electrodes for stimulation and recording the muscle action potentials were made of platinum wire 1 mm in diameter. In some of the experiments, pairs of 5 mm wide platinum plates were also used for holding the preparations and recording the electrical activity of muscle (Biró, 1975). There was no significant difference in the action potentials recorded with the two kinds of electrodes. The figures give detailed illustration of the connections between the electrodes and the preparations.

The action potentials were recorded by oscillographic method using two or three channels of the oscilloscope. In the cases of the simultaneous records, the upper and the middle beam of the oscilloscope displayed the action potentials of the first and the second muscles, respectively. The lower beam was used for recording the stimulus as a point above the time markers of 1 ms.

The experiments were carried out at room temperature (21 – 23 °C) the preparations were kept under wet condition.

Results

At the beginning of this series of experiments it was demonstrated that the magnitude of the secondary action potential depends mainly upon the position of the second nerve on the dorsal surface of the first muscle (Fig. 1). When this nerve was situated in lateral positions (Fig. 1a and b) or in a wavelike form (Fig. 1c) the action potential of the second muscle appeared with much less amplitude where the nerve was laid along the middle line of the first m. gastrocnemius (Fig. 1d).

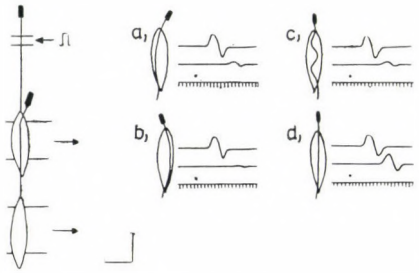

Fig. 1. Simultaneous records of action potentials from the first and the second muscles in the cases of laying the second nerve in two lateral (a and b), wavelike (c) and straight line (d) positions on the first muscle. Time markers on the lower beam is 1 ms in all of the figures. Calibration: 100 mV — 10 ms

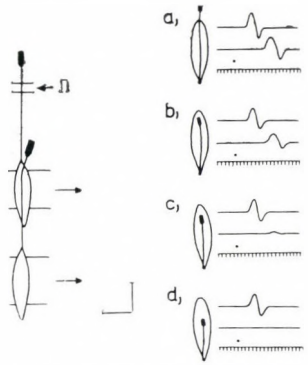

Fig. 2. Simultaneous records of action potentials from the first and the second muscles in the cases (a—d) of shortening the length of the nerve laid on the first muscle. Calibration: 100 mV—10 ms

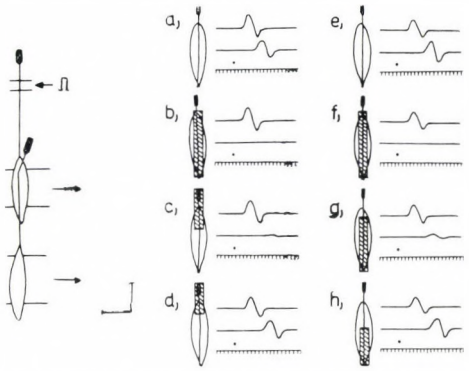

Fig. 3. Simultaneous records of action potentials from the first and the second muscles in different cases of insulating the first muscle from the second nerve. a and e: no insulation; b and f: complete insulation: c and d: partial insulation after moving the nylon foil in proximal direction; g and h: partial insulation after moving the nylon foil in distal direction. Calibration: 100 mV—10 ms

If we changed the length of the nerve connected with the muscle the transmission of excitation from muscle to nerve also modified (Fig. 2). Shortening the connection resulted a progressive decrease in the amplitude of the action potential recorded from the second muscle (Fig. 2b and c) and the connection in the distal half portion of the muscle was not sufficient for an effective stimulation of the nerve (Fig. 2d).

Alterations in the connection between the nerve and the muscle were accomplished by means of a 0.1 mm thick nylon foil (Fig. 3). Complete insulation prevented the muscle from stimulating the nerve (Fig. 3b and f). Moving the nylon foil in proximal and distal directions made it possible for muscle to exert its stimulating effect on the nerve. The minimal response of the second muscle appeared when the nerve contacted with it in about 60 per cent (Fig. 3c) and

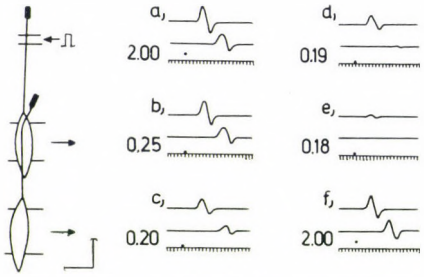

Fig. 4. Simultaneous records of action potentials from the first and the second muscles after exciting the first nerve with the stimuli of different amplitude. a) 2V, b) 0.25 V, c) 0.20 V. d) 0.19 V, e) 0.18 V, f) 2 V. Duration of stimuli was 0.1 ms. Calibration: 100 mV—10 ms

about 15 per cent (Fig. 3g) of the muscle length in the distal and the proximal parts of the muscle, respectively. Further movement of the nylon foil in both directions brought about the appearance of the secondary muscle action potential with maximal amplitude (Fig. 3d and h).

The results shown in the previous two figures (Figs 2, 3) demonstrated that, in the case of the m. gastrocnemius, the proximal part of the muscle could play a more significant role than the distal one in the transmission of excitation from muscle to nerve.

This transmission of excitation is greatly influenced by the magnitude of the electrical activity generated in the first muscle, too (Fig. 4). The effects of the different muscle activities on the nerve are demonstrated by the oscillograms showing the secondary responses produced by the electrical activities of the first muscle if its nerve was excited with supramaximal (Fig. 4a and f), maximal (Fig. 4b), two different submaximal (Fig. 4c and d) and threshold (Fig. 4e) stimuli. These records demonstrate that the diminution in the secondary responses has been brought about by the decrease in the electrical activity of the first muscle.

The secondary action potentials of different sizes were also obtained in the experiments in which the whole leg preparations were used as starting preparations. The nerve of the second preparation was laid on the abdominal surface of the thigh, in this way more than one muscle could have an excitatory effect on the nerve. When the position of the nerve on the femoral muscles changed, their excitation produced different action potentials recorded from the muscle of the second preparation.

According to all the former results, the secondary responses of different sizes could be produced by various electrical activities of the muscles or muscle fibres in contact with the second nerve. Therefore experiments were performed on nerve-muscle preparations for modelling the joint effects of the muscles on formation of their compound action potentials. The results obtained in these experiments correspond to the summation of the electrical signals.

When the two preparations were excited with separate and simultaneous stimulation, the common electrical activity of muscles were recorded in four

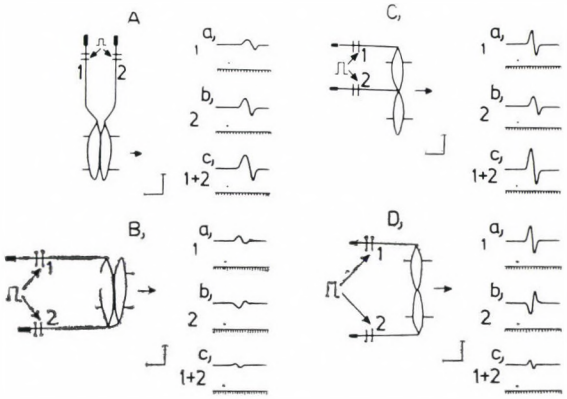

Fig. 5. Action potentials recorded from two muscles after stimulating their nerves separately (a: 1, b: 2) and simultaneously (c: 1+2). The muscles were in parallel (A and B) and in series (C and D) connections in the cases of travelling their excitation in the same (A and C) and opposite (B and D) directions. Calibration: 100 mV—10 ms

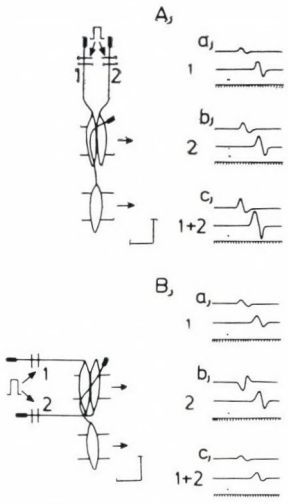

Fig. 6. Muscle action potentials recorded from the primary and the secondary preparations in the cases of laying the secondary nerve on two primary muscles which propagated their excitation in the same (A) and opposite (B) directions. The stimuli were given to the primary nerves separately (a: 1, b: 2) and simultaneously (c: 1+2). Calibration: 100 mV—10 ms

different arrangements of the preparations (Fig. 5). In the first two cases the muscles were in parallel connection and their excitation travelled in the same (Fig. 5A) and in the opposite (Fig. 5B) direction. In the second two cases the muscles were connected to each other in series and their excitations also travelled in the same (Fig. 5C) and in the opposite (Fig. 5D) direction. The oscillograms

obtained in these experiments show that by travelling the excitation in the same and opposite directions, the common responses of the preparations were respectively larger (Fig. 5A and C) and smaller (Fig. 5B and D) than the individual responses.

Based on the previous results, the joint effects of two muscles were also investigated in connection with the transmission of excitation from muscle to nerve (Fig. 6). In this case the muscles could individually produce different secondary responses and the simultaneous excitation of the muscles brought about a secondary action potential larger than the individual ones (Fig. 6A). When the excitation of the primary muscles spread in opposite direction, the secondary action potential was smaller after the simultaneous stimulation than in the cases of the individual stimulations (Fig. 6B).

In connection with the cases in which the secondary nerve was subjected to the effect of the electrical activity of two muscles, it can be noted that changes in the positions of the nerve on the primary muscles could also cause differences ni the secondary responses.

Discussion

It has been demonstrated that one of the significant factors in the transmission of excitation from muscle to nerve is the anatomical relation between the two excitable tissues. The experimental results illustrated in Figs 1–3 have proved that the production of the secondary action potentials is determined by the size and the position of the area through which the muscle could exert its stimulating effect on the adjacent nerve. It has been found that, in the case of the dorsal surface, the proximal part of the m. gastrocnemius is of greater importance for the transmission of excitation than the distal part of the muscle. This finding is consistent with the results obtained under in situ circumstances (Biró, Vu-Duy-Thinh, 1977). The fact that the proximal part of the m. gastrocnemius has greater significance than the distal one could be explained on the basis of the structure constructed by the fibres in this muscle.

There are several papers in which observations have been published on the different forms of the nerve excitation generated by muscle activities. According to these the nerve impulses induced by muscle fibres inside the muscle could be recorded from the ventral root (Masland, Wigton, 1940; Eccles et al., 1942; Lloyd, 1942; Leksell, 1945; Brown, Matthews, 1960a) and from the sensory (Granit et al., 1959; Sumner, 1975) and motor nerves (Werner, 1961; Epstein, Jackson, 1970).

Under the physiological conditions the peripheral nerves are in the vicinity of the muscles or muscle fibres. As a consequence of this situation the nerves are subjected to the electrical activities travelling in different directions on the muscles connected to each other in parallel and in series. In addition to these factors the temporal relations of the muscle activities also have to be involved in determining the instantaneous electrical effects on the nerves.

It is illustrated in Figs 4 and 6, that the excitatory effect of the muscles on the adjacent nerves considerably depends upon the magnitude of the compound action potential of muscles. Therefore, it would be important to know the mechanism of compounding the electrical activity generated in the different motor units. Our results shown in Fig. 5 point to a certain summation of the muscle action potentials which was also studied by Brown and Matthews (1960b), Moore (1967), Biró and Partridge (1971) and Lee et al. (1975).

As a consequence of the complex electrical, anatomical and temporal relations of the excitatory processes, in some cases, the magnitude of the single or compound action potentials of muscles could not be large enough for eliciting excitation in the nerve in contact with muscles. In these cases, however, the excitatory influence of the muscle on the nerve could also manifest itself in subthreshold effects.

The excitatory and the presumed subthreshold effects of the muscles on the adjacent nerves correspond to the results published in the papers which treat the transmission of excitation between nerve fibres (Hering, 1882; Jasper, Monnier, 1938; Rosenblueth, 1941; Arvanitaki, 1942) and the excitability changes evoked in a resting nerve fibre by the activity of a neighbouring one (Blair, Erlanger, 1940; Katz, Schmitt, 1940; Marrazzi, Lorente de Nó, 1944; Clark, Plonsey, 1971; Markin, 1973; Katalymov, 1974).

A deeper understanding of how the electrical activity of the different muscles may affect the adjacent nerves also requires further investigations.

The results presented in this paper together with other authors' findings mentioned above support the hypothesis (Biró, 1975) that the effects of the electrical muscle activity on the nerve in contact with the muscle could play an additional role in the regulating mechanisms which take place in the excitatory processes concerning the nerve and muscle functions.

The advice of Professors E. Ernst and J. Tigyi are gratefully aknowledged.

References

Arvanitaki, A. (1942) J. Neurophysiol. *5* 89—108
Biró, G. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. *10* 287—295
Biró, G. (1977) Acta Biochim. Biophys. Acad. Sci. Hung. *12* 279—282
Biró, G., Partridge, L. D. (1971) J. Appl. Physiol. *30* 521—526
Biró, G., Vu-Duy-Thinh (1977) Acta Biochim. Biophys. Acad. Sci. Hung. *12* 57—70
Blair, E. A., Erlanger, J. (1940) Am. J. Physiol. *131* 483—493
Brown, M. C., Matthews, P. B. C. (1960a) J. Physiol. *150* 332—346
Brown, M. C., Matthews, P. B. C. (1960b) J. Physiol. *151* 436—457
Clark, J. W., Plonsey, R. (1971) Biophys. J. *11* 281—294
Eccles, J. C., Katz, B., Kuffler, S. W. (1942) J. Neurophysiol. *5* 211—230
Epstein, R. A., Jackson, S. H. (1970) J. Appl. Physiol. *28* 407—410
Granit, R., Pompeiano, O., Waltman, B. (1959) J. Physiol. *147* 399—418

Hering, E. (1882) Sitzungsberichte der mathematisch-naturwissenschaftlichen Classe der kaiserlichen. Akademie der Wissenschaften *85* 237—275
Jasper, H. H., Monnier, A. M. (1938) J. Cell Comp. Physiol. *11* 259—277
Katalymov, L. L. (1974) Physiol. J. USSR *60* 1518—1525 (in Russian)
Katz, B., Schmitt, O. (1940) J. Physiol. *97* 471—488
Lee, R. G., Ashby, P., White, D. G., Aguayo, A. J. (1975) Electroencephal. Clin. Neurophysiol. *39* 225—237
Leksell, L. (1945) Acta Physiol. Scand. *10* Suppl. XXXI. 1—84
Lloyd, D. P. C. (1942) J. Neurophysiol. *5* 153—165
Markin, V. S. (1973) Biophysics *18* 314—321 (in Russian)
Marrazzi, A. S., Lorente de Nó, R. (1944) J. Neurophysiol. *7* 83—101
Masland, R. L., Wigton, R. S. (1940) J. Neurophysiol. *3* 269—275
Matteucci, C. (1842) cited in: Handbook of Physiology. Ed.: J. Field, Section 1: Neurophysiology, American Physiological Society, Washington, D. C. 1959. Vol. 1. p. 20
Moore, A. D. (1967) Am. J. Phys. Med. *46* 1302—1316
Rosenblueth, A. (1941) Am. J. Physiol. *132* 119—128
Sumner, A. J. (1975) J. Physiol. *246* 277—288
Werner, G. (1961) J. Neurophysiol. *24* 401—413

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An Influence of the Extracellular Redox State on Ion Flux of D-Type Giant Neurones of *Lymnea stagnalis*

(Preliminary Communication)

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To examine the ion mechanism of the redox phenomenon described by us earlier (Puppi et al., 1968, 1975, 1976) recently we analyzed the ion currents through the cholinergic, electrogenic and/or leakage current channels of the membranes of giant neurones as they were modified by the extracellularly applied redox agents. Our objective was light on the finer mechanisms through which the redox phenomenon is manifested.

Abbreviations used

Ach	— acetylcholine
ASC	— ascorbic acid
[C]	— control
ΔV_{Ach}	— depolarization caused by Ach
E_0'	— redox state potential at pH 7
E_e	— reverse potential
$E_{e(\text{ch})}$	— reverse potential of cholinergic channels
E_m	— membrane potential
E_r	— resting potential
G	— conductance
G_{ch}	— conductance of cholinergic channels
G_e	— conductance through electrogenic channels
G_L	— conductance through leakage current channels
MB	— methylene-blue
$R_{s(i)}$	— slope resistance of input currents
$R_{s(o)}$	— slope resistance of output currents
RSP	— redox state potential
[T]	— date following redox agent administration

Our experiments were carried out on completely isolated giant neurones of *Lymnea stagnalis* (Kostenko, 1972). Randomly selected cells from the visceral and great parietal ganglia were used. The three-electrode method of Voltage Clamp was applied. Acetylcholine was introduced iontophoretically through an electrode moved by separate micromanipulator. Applications of redox agents (10^{-4}M) were made through the perfusion chamber in which the cells were kept.

The following redox agents were used: A) reductants: ascorbic acid, p-methyl-amino-phenolsulphate, hydrochinon, $CoCl_2$. B) oxidants: methylene blue, thionine, para-aethoxy chrisoidine, $CuCl_2$.

The experiments showed that:

I. The E_m *values* increased on the averages by 3 mV following the application of reductants but were reduced by oxidants to the extent of 6 mV.

II. The G_{ch} *values*, as seen on Fig. 1 got decreased slightly by oxidants but strongly by reductants.

III. The $E_{e(ch)}$ *values* changed inversely. In the presence of oxidizing agents the E_e was shifted to more negative values of E_m on the potential axis, while the application of reductants shifted this parameter to more positive E_m.

IV. The ΔV_{Ach} *values* altered also inversely. The oxidants increased this parameter (Fig. 1) but reductants usually caused a significant diminution in the measure ot the depolarization caused by Ach.

V. Fig. 2 shows the correlations of the slope resistance values of fluxes through *electrogenic channels* as plotted against the redox state brought about by redox agents. The points of the coordinates of slope resistances were calculated as follows:

$$\frac{R_{s(o)}[C]}{R_{s(o)}[T]} \cdot 100\,\% - 100 \tag{1}$$

for the outward current and

$$\frac{R_{s(i)}[C]}{R_{s(i)}[T]} \cdot 100\,\% - 100 \tag{2}$$

for the inward current.

The slope resistance of outflux currents displays a monotonic decrease parallel with the growing E_o'. It suggests that the amount of the outflowing K^+ ions is directly proportional to the RSP.

In the case of the $R_{s(i)}$ there are peak-type correlations between the G-s through influx channels and the actual E_o' in the biophase. This indicates that for the maximal inward $Na^+ - Ca^{++}$ flux there exists an optimal E_o'value.

VI. When analysing the influence of the E_o' on the resistance of the *leakage current* we observed that in the reducing range this parameter was diminished almost exponentially by the growing E_o. In the oxidizing range this diminutional tendency started again but from a higher level than the last value produced by the reductants, in other words a break was observed in the exponential curve at the border of the effects of the reducing and/or oxidizing agents. In suggests

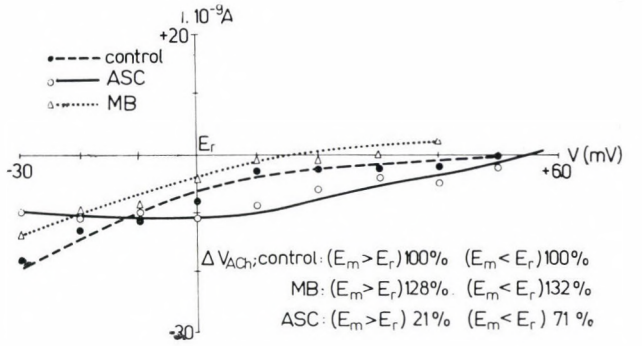

Fig. 1. Voltage/ampère characteristics of ion flux through cholinergic channels following the extracellular application of MB and ASC

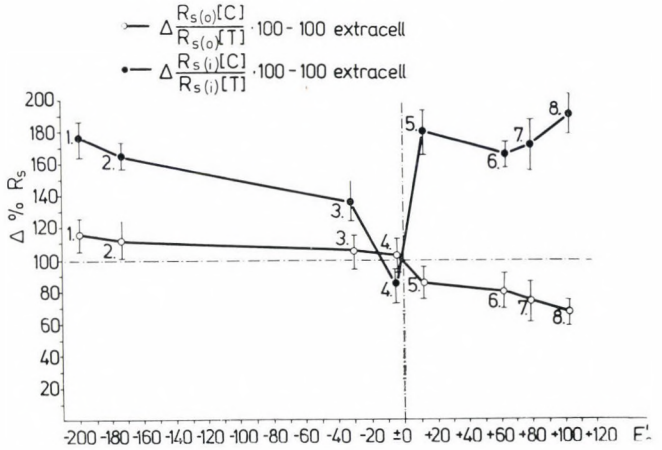

Fig. 2. Slope resistance values of the input ($R_{s(i)}$ and the output ($R_{s(o)}$) currents following the extracellular application of redox agents as they were plotted against the E_o' brought about by redox agents (in mV). Redox agents: 1) ascorbic acid, 2) para-methyl-amino-phenol-sulphate 3) hydrochinon, 4) $CoCl_2$ (reductants) and 5) methylene-blue, 6) thionin, 7) para-aethoxy-chrisoidine, 8) $CuCl_2$ (oxidants)

that the oxidizing and/or reducing agents act on different ligands of the receptors in the functional vicinity of G_L channels.

Considering that such important parameters of ion transport (e.g. G_{ch}, E_e, G_e and so on,) result significant alterations depending upon the actual RSP of the biophase, the following conclusions can be drawn:

I. In the excitable systems exists a fairly complicated regulatory mechanism too which consists of : 1) State and/or changes of metabolical E_0' values in the cytoplasm, the membrane and/or the intercellular spaces and 2) excitable systems

which are sensitive to the actual state and/or changes of the former state. These sensitive sites may be the following: a) conformations of proteins of the channels (Webb, 1966; Koltover, 1973), b) changes of fixed charges in the membrane (Passow, 1969), c) alterations in the degree of lipid peroxidation, which also influences the G values (Hunter et al., 1963) and last but not least, d) the active transport processes (Dick et al., 1969; Dikstein, 1971; Wald et al., 1972; Puppi et al., 1975).

II. The level of E_o' – similarly to the pH values – has to be standardized or at least measured in every experiment to receive reliable basis for comparison of the different experimental data.

III. It is known that in stress situations due to the increased metabolism there is an enhancement of oxidation (an elevation in E_0'). Though it means an exhaustory tendency – in other words a decreased ability of living organisms to accomodate to the increased demands – the oxidation offers a newer grade of accomodation at the level of excitation by mobilizing newer resources to counteract the grading exhaustion.

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References

Dick, D. A. T., Dick, E. G., Tosteson, D. C. (1969) J. gen. Physiol. *54* 123—129
Dikstein, S. (1971) Naturwissenschaften *58* 439—443
Hunter, F. E., Jr., Gebicki, J. M., Hoffstein, P. E., Weinstein, J., Scott, A. J. (1963) J. Biol. Chem. *238* 828—838
(Kostenko, M. A.) (1972) Цитология, *14* 1274—1280
(Koltorev, V. K.) (1973) Биофизика, *18* 661—665
Passow, H. (1969) In: Progress in Biophysics and Molecular Biology, 19. Part II. Pergamon Press, London
Puppi, A., Tigyi, A., Szalay, L. (1968) Acta Biol. Acad. Sci. Hung. *19* 517—518
Puppi, A., Szalay, L., Dely, M. (1975) Comp. Biochem. Physiol. *50 C* 75—79
Puppi, A., Szalay, L., Dely, M. (1976) Acta Biochim. Biophys. Acad. Sci. Hung. *11* 63—73
Wald, C., Czaczkes, W., Dikstein, S. (1972) Naturwissenschaften *59* 316—317
Webb, J. L. (1966) Enzyme and metabolic inhibitors, Vol. II. Malonate, analogs, dehydroacetate, sulfhydryl reagents, i-iodosobenzoate, mercurials. Academic Press, New York and London

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Investigations of the Uptake of Antibiotics Influencing Membrane Function by *E. coli* Cells

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The antibiotic-uptake (polymixin, gramicidin, nigericin, streptomycin) of *E. coli* cells was studied in relation to the antibiotic concentration of medium, the time and the temperature. The time-dependence is illustrated by a curve consisting of two periods in the case of polymixin and gramicidin, and by a curve consisting of three periods in the case of nigericin and streptomycin in the interval under examination. The initial quick uptake of a few minutes is followed by a saturation (plateau) in all four cases. In the latter period the concentration-dependence of the uptake is characterized by a curve approaching saturation for each of the examined antibiotics. On the basis of our experimental results the primary process of the interaction between the surface of the bacterium and the antibiotic is interpreted as an adsorption process. On the basis of this model the average number of the possible binding sites on the surface of the bacterium is $\sim 10^6$, which corresponds well to values determined by others in other ways. The adsorption-equilibrium constants and adsorption energies in the case of the examined antibiotics were also determined. The latter values (3300 – 7500 cal/mole) show a weak interaction – as it was expected.

A basic problem of the effect of antibiotics on bacteria is the passage of antibiotics into the bacterium cell, in which the interaction between the antibiotic molecule and the bacterial surface has a primary role. The interaction between antibiotic molecules and the binding sites on the bacterial surface (possibly peripheral amino-acid residues of proteins, polar and apolar groups of lipids) can be realized through Coulomb – van der Waals forces, by formation of hydrogen bridge, hydrophobic bonds (Allison, 1968; Vandenheuvel, 1971; Vransky, 1975). The binding sites of some antibiotics in the cell were determined with radioactive tracer method and other methods (NMR, ESR spectroscopy) (Mc Laughlin, Eisenberg, 1975; Teuber, 1973; Gale et al., 1972; Pache et al., 1972; Gorini, 1976; Hancock, 1964). It was e.g. determined by experiments with *E. coli* and its membrane fraction resp., that polymixin interacts with the phospholipid components of the membrane (Barrett-Bee et al., 1972; Bader, Teuber, 1973; Teuber et al., 1974).

The aim of our investigations was to study the primary interaction between the bacterial surface and the molecules of antibiotics. Similar examinations had

been performed formerly with streptomycin (Szőgyi et al., 1969). Our experiments were extended now to three further antibiotics (polymixin, gramicidin and nigericin) and their uptake was studied in relation to the antibiotic concentration of the medium, the time and the temperature. The results of the recent experiments were interpreted with the aid of the model worked out for streptomycin, thus it became possible to deduce some quantitative conclusions.

Methods

Polymixin B sulfate, gramicidin and nigericin were used in our investigations. The experiments were performed with *Escherichia coli* B. strain. The cells were grown in bouillon medium at 37 °C for a night. The bacteria were inoculated from this subculture into 1000 ml of bouillon before beginning the experiments. The bacteria in the logarithmic phase of growth were centrifuged (8,000 turns/minute for about 30 minutes) then they were incubated in a bouillon medium of an antibiotic concentration of $10-40$ μg/ml. After the time of incubation the medium containing antibiotics was removed by centrifugation (30,000 turns/minute for about 5 minutes). The microbes were washed with bouillon, then centrifuged again (30,000 turns/minutes for about 5 minutes). After that the bacteria were suspended in 0.4 ml of sterile distilled water. From this suspension 0.1 ml was used in each case for the determination of the quantity of antibiotic taken up by bacteria, which was carried out by the agar-diffusion method (Szőgyi, Tamás, 1969). 3×0.05 ml of the suspension was used for the determination of viable counts (series dilution method). The viable cell counts were $10^9 - 10^{10}$ bacteria/ml at an average.

Results

In Fig. 1 the time of incubation is indicated on the horizontal axis, and the average number of polymixin molecules taken up by bacteria is indicated on the ordinate. It can be seen that the rate of uptake of molecules decreases gradually, and after about 20 minutes uptake hardly changes (plateau period). Concentrations of polymixin greater than 20 μg/ml as well as time of incubation longer than 40 minutes were not used in our experiments, because a decrease in the number of viable cells was experienced in these cases. Uptake curves similar to the above ones were obtained also in the case of *gramicidin* (Fig. 2). (Gramicidin had no bactericidal effect in the applied concentration even after an incubation of 60 minutes.)

The time-dependence of *nigericin* uptake is shown by Fig. 3. The quantity of the nigericin uptake increases through about 10 minutes, then it scarcely changes for about 40 minutes (plateau), then the uptake increases again. If the outer concentration is increased, the curves shift toward higher uptake values, but it

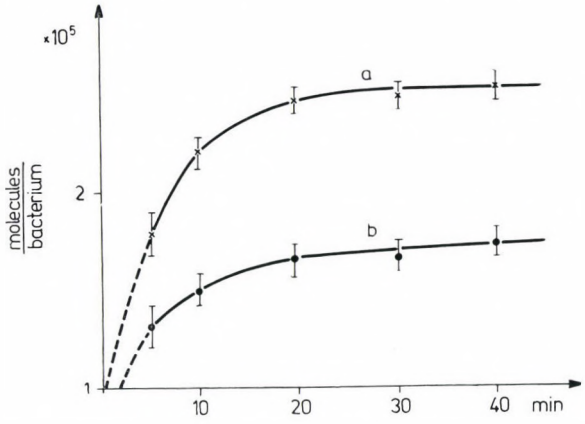

Fig. 1. Time-dependence of polymixin uptake of *E. coli* cells. *a* belongs to an antibiotic concentration of 20 μg/ml, and *b* belongs to one of 10 μg/ml. One point means the average of ten experiments

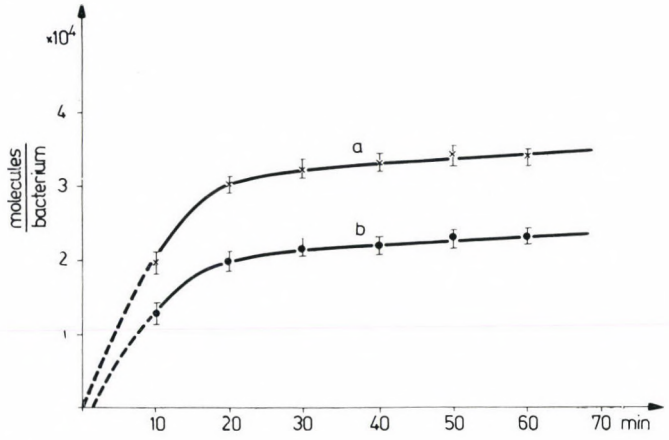

Fig. 2. Time-dependence of gramicidin uptake of *E. coli* cells. *a* belongs to an antibiotic concentration of 70 μg/ml, and *b* belongs to one of 50 μg/ml. One point means the average of eight experiments

can be also observed, that meanwhile the plateau periods become shorter, and the third period becomes steeper and steeper.

The curves in Figs 1 and 2 have two periods, while those in Fig. 3 have three one. An uptake curve of three periods was also obtained in the case of *streptomycin* (Szőgyi et al., 1969). In Fig. 4 the average number of polymixin (curve 1), nigericin (curve 2) and gramicidin (curve 4) molecules taken up by one bacterium was plotted against the antibiotic concentration of the medium in the case of

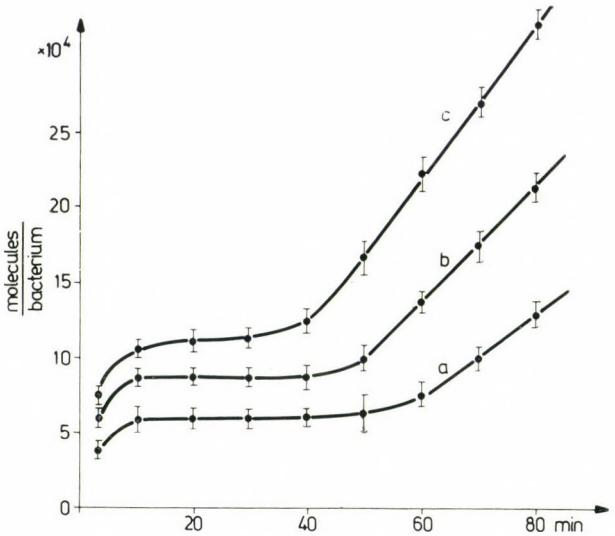

Fig. 3. Nigericin uptake of *E. coli* cells. *a* belongs to a nigericin concentration of 20 μg/ml, *b* belongs to one of 30 μg/ml, and *c* belongs to one of 40 μg/ml. One point means the average of eight experiments

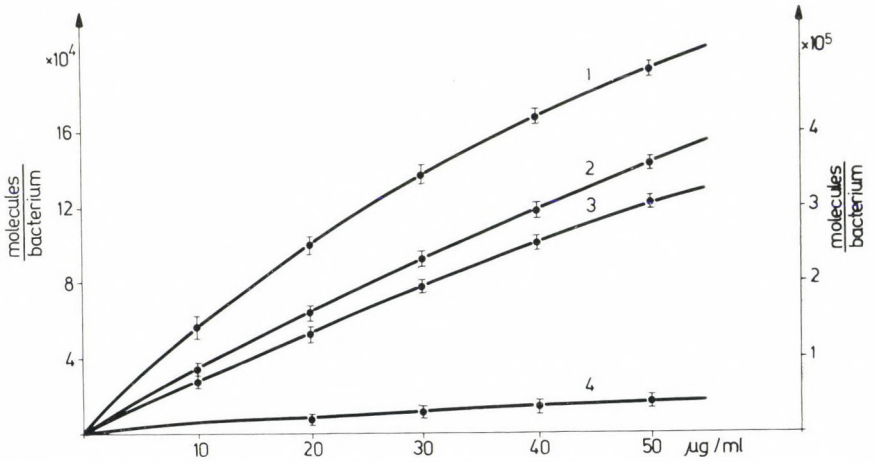

Fig. 4. Polymixin (1), nigericin (2), streptomycin (3), gramicidin (4) uptake plotted against the antibiotic concentration of the medium in the case of an incubation of 20 minutes. The right ordinate belongs to curve 1, the left one belongs to curves 2—4. One point means the average of ten experiments

an incubation of 20 minutes. The incubation time was chosen so that it fell to the horizontal (plateau) period of the uptake-curves of Figs 1–3 in each case. The results of the preceding experiments performed with streptomycin are also shown in the figure (curve 3). The number of bound molecules is especially great in the case of polymixin (order of magnitude: 10^5/bacteria), nigericin and streptomycin uptake is about an order of magnitude smaller than this. In the experiments with gramicidin a smaller uptake was experienced even in concentrations greater than the above ones (25–75 μg/ml).

Discussion

The uptake curves shown in Figs 1–4 indicate, that the first step of the interaction between the antibiotic molecule and the surface of bacterium cell can be interpreted as *adsorption*. (Streptomycin uptake was treated in this sense in the preceding paper.) The quickly rising period of the uptake is in connection with the ever increasing adsorption of the molecules. The setting in of the dynamic equilibrium of adsorption-desorption processes is characterized by an almost horizontal period of the curve (plateau). The third period, which can be observed only in the case of streptomycin and nigericin indicates that the above-mentioned antibiotic molecules get into the cell through the function-damaged membrane. But the latter period is missing, if the antibiotic is bound to membrane components, as it was experienced in the case of polymixin and gramicidin.

All of the curves in Fig. 4 can be described with Langmuir's law of adsorption:

$$N_A = \frac{C_A N}{\kappa + C_A}, \tag{1}$$

where N is the average number of the possible binding sites on the surface of one bacterium and N_A is the average number of the places occupied by the antibiotic molecules in the adsorption-desorption equilibrium, C_A is the antibiotic concentration of the medium, κ is the equilibrium constant of adsorption belonging to the given temperature and experimental conditions. κ shows the relation between the free and occupied places in the case of unit antibiotic concentration in the medium. Thus the reciprocal of κ can be considered as the measure of the "affinity" of the antibiotic to the membrane. C_A and N_A are directly known from measurements, and the values of N and κ can be determined from the comparison of relation (1) with the curves of Fig. 4. The weighted smallest squares method was used in the determination. (The function minimum place was obtained with a Hewlett-Packard computer with a gradient method.) The points of measurement in Fig. 4 were connected to give a curve gained already from this fitting. According to our measurements the average number of possible binding sites on the surface of one *E. coli* bacterium is $\sim 10^6$ (more exactly: $(0.9-1.3)\times 10^6$/bacterium), which agrees well with the value determined by others in an other

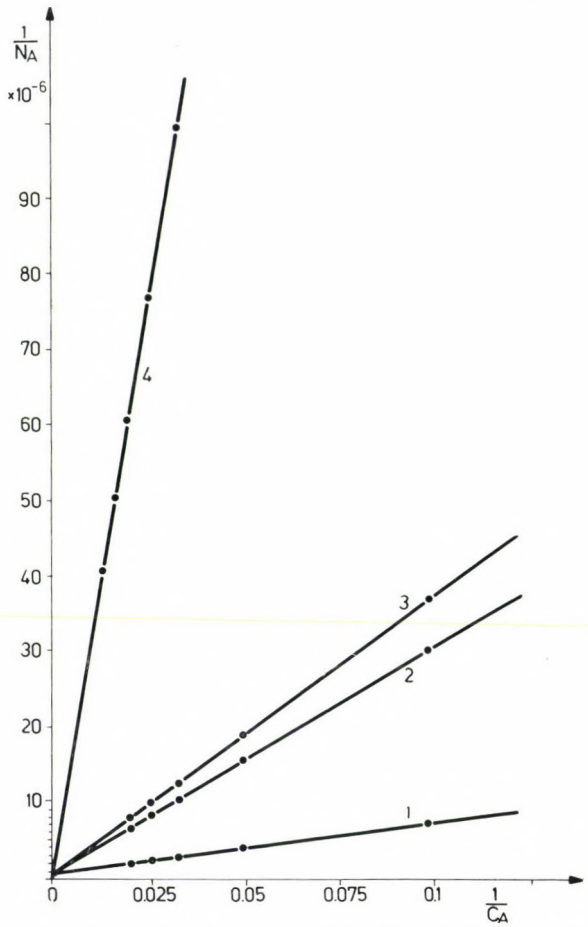

Fig. 5. The reciprocal of the number of polymixin (1), nigericin (2), streptomycin (3) and gramicidin (4) molecules taken up by one microbe (N_A) plotted against the reciprocal of the antibiotic concentration of the medium (C_A)

way (Teuber, 1973; Bader, Teuber, 1973.). The value of N can be also demonstrated by the reciprocal representation of the data of measurements of Fig. 4 with the method of Lineweaver and Burk (Fig. 5). In this case the intersection shows the value of N.

Table 1 contains the equilibrium constants of the antibiotics examined by us and determined at room temperature. The value belonging to polymixin is the smallest, thus the "affinity" of polymixin to the membrane is the greatest. The "affinity" of nigericin and streptomycin is smaller than that of polymixin. The adsorption equilibrium constant of gramicidin is an order of magnitude greater than the preceding one, thus its "affinity" to membrane is the smallest of the four antibiotics.

Table 1

Adsorption-equilibrium constants and adsorption energies of the examined antibiotics

Antibiotic	κ $\times 10^{-4}$ g/ml	E_{ads} cal/mol
Polymyxin	0.7± 0.01	7500
Nigericin	3.0± 0.03	5200
Streptomycin	3.6± 0.04	4800
Gramicidin	38.0± 0.5	3300

The adsorption equilibrium constants of the examined antibiotics were determined also at 5 °C and 37 °C beside room temperature (20 °C). With the knowledge of these data the adsorption energy values were calculated, which are also shown in Table 1. The greater the "affinity" of the molecule to the membrane is, the higher is the binding energy of the receptor site to the antibiotic. Adsorption energy values indicate a weak interaction between antibiotic molecules and the membrane, as it had been expected.

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References

Allison, A. C. (1968) British Medical Bulletin *24* 135
Bader, J., Teuber, M. (1973) Z. für Naturforschung *28* 422
Barrett-Bee, K., Radda, G. K., Thomas, N. A. (1972) Interactions, perturbations and relaxations of membrane bound molecules. In: FEBS Proceedings of the Eight Meeting. Amsterdam *28* 231
Gale, E. F., Gundliffe, E., Reynolds, P. E., Richmond, M. H., Waring, M. J. (1972) The molecular basis of antibiotic action. Wiley — Intersci. Publ. London, New-York, Toronto, p. 121
Gorini, L. (1976) Fed. Proc. *5* 26
Hancock, R. (1964) J. Bact. *88* 633
Mc Laughlin, S., Eisenberg, M. (1975) Ann. Rev. Biophys. Bioeng. *4* 335
Pache, W., Chapman, D., Hillaby R. (1972) Biochim. Biophys. Acta *255* 358
Szőgyi, M., Tamás, Gy., Tarján, I. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 415
Szőgyi M., Tamás Gy., (1969) Gyógyszerészet *13* 452
Teuber, M. (1973) Z. für Naturforschung *28* 476
Teuber, M., Bader, J., Schindler, P., Bowman, B. F. (1974) Zbl. Bakt. *228* 243
Vandenheuvel, F. A. (1971) Advances in lipid research *9* 225
Vransky, V. K. (1975) Surface electric properties of cellular membranes. In: Biophysics of membrane transport (ed.: J. Gomulkiewicz, B. Tomicki) *2* 240

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Elementary Biological Units: Biocomplexes and Organoids*

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It is timely to raise the questions: where are we today in understanding animate matter and what will be the right course life science has to pursue in the future. Such reflections suggest the situation of a century ago, when striving to overcome the emptiness of "Lebenskraft" (= Glisson's "vital force") J. Müller (1837), C. Ludwig, J. R. Mayer (both 1842) E. Brücke, E. du Bois Reymond and H. Helmholtz produced a "storm period" in German physiology. But all their most valuable achievements did not result in complete success – according to A. Fick (1874). Consequently, abandoning this trend of "exact" bioscience, the following research workers turned toward "living" problems, e. g. vivisection, or morphology and produced very valuable results. However, neither these nor the brillant discoveries in biochemistry and in newly revived biophysics seemed to satisfy Schrödinger's wondering "what is life" (1944), and even today, a century after Fick's statement, we are not very much further.

Thus, without neglecting investigations being used today by biochemistry (in test-tubes) and biophysics (e. g. on lipid bilayers) I should like to emphasize the importance of research work on "living" objects, to return to the cell being "a fundamental structural and functional unit of living organisms, just as is the atom in chemical structure" (De Robertis et. al., 1965). The comparison is, indeed, very proper: in contrast to the quotation, both are not real functional and structural elementary units but very complicated formations, the cell containing many simple separate units of the bioplasm (biochomplexes, organoids), and the atom being composed of the elementary particles. Now, it should be remembered that basic scientific understanding of chemical processes had not become possible until the real *elementary particles* (electron, proton, neutron) and their activities during the processes were taken into account. Accordingly, some *biological elementary units* producing *elementary biological functions* similar to those taking place inside the cell should be investigated.

1. As to the biological elementary units, firstly the *biocomplexes* should be mentioned. Being isolated these biocomplexes are sometimes but "models" of the components in the cell, notwithstanding "it seems that models at every level can be useful" (Eichhorn, 1973). At any rate, to understand the functions of

* Study for the lecture at the symposium organized at the Congress of the Hungarian Biochemical, Biophysical and Physiological Societies, Pécs, June 30, 1977.

biocomplexes *the state of their water and inorganic contents* should be clarified.

a) Considering *biowater* in its general importance its state in the cell should be known; the difficulty of the situation is very clearly shown by this quotation (De Robertis et. al. 1965): "Water in the cell is in two forms: free and bound. *Free water* represents 95 per cent of the total cellular water and is the part mainly used as solvent for solutes and as a dispersion medium of the colloid system of protoplasm. *Bound water*, which represents only 4 to 5 per cent of the total cellular water, is loosely held to the proteins by hydrogen bonds and other forces. It includes the so-called unmobilized water contained within the fibrous structure of macromolecules."

Disregarding the shortcomings in this quotation neglecting the *boundedness of water molecules* as ligands, part of chelates or clathrates etc., the general characterization of free and bound water ought to be stated precisely. E. g. in saturated solutions of KCl or NaCl the ions are hydrated by $\sim 4-8$ molecules of H_2O, and this water can be considered as "*bound*", since the hydration shells are known to migrate together with the ions in an electric field. In the saturated KCl solution glucose, in the saturated NaCl solution* urea can dissolve in the amount of saturation demonstrating that this *bound water can play the role of solvent.*

A survey of the watery systems indicates that the opinion looking upon their water content as a *passive medium* for the activities of the soluted particles – similarly to the vacuum for the particles of gases – is unfounded and mistaken. This statement is valid to a greater extent for biological systems and their elementary units mentioned above: their water content is in close connection with their functions: *biowater is not a passive medium for biofunctions but participates actively in them.* And that is the innermost essence of our discussions of the water content of biological systems. This aspect** gained ground in the last two decades by the works of Troshin (1956, 1966) and Ling 1972), furthermore in papers of many other authors (see e. g. Hazlewood, 1973).

b) A similar history could be described concerning the problem of "*bound*" *inorganic atoms or ions contained in biocomplexes.* More than half a century ago I performed experiments indicating (Ernst, Scheffer, 1928) "boundedness" of muscle potassium; later*** I continued experimental research work in this direction in collaboration with co-workers (see e. g. Tigyi, 1959). Referring to many newer publications I lay stress as well on two other books describing biocomplexes and strongly corroborating the conception concerning "bound" inorganic atoms or ions and their activities being most likely indispensable in biological processes.**** (Hazlewood, 1973; Eichhorn, 1973).

* The question of crystals containing NaCl and glucose or urea is outside the scope of this paper.

** The functional role of water e.g. in muscle was emphasized by Engelmann (1878) and expounded by us in some papers, the first one (Ernst, 1928) describing the role played by "water migration as the basis of contraction". Such an aspect of biowater in muscle was accepted also by Szent-Györgyi (e.g. 1957).

*** A paper of Hill (1930) described contradictory statements, and I published a paper (Ernst, 1934) refuting Hill's data; after that Hill did not return to this problem.

2. When considering the biocomplexes as "models", one has the circumstance in view: "A fundamental question . . . is whether it is useful to study the model when the 'natural' substance is at hand. To answer this question it is required to make a realistic appraisal of the ways in which biological mechanisms are elucidated. The living cell is so complex that its workings can be understood only by isolating component parts, always with the inherent risk that the parts in isolation do not function as they do in the cell"(Eichhorn, 1973).

In this sense the chemical and physical[1] research work on the morphological units of the cell comes into prominence: *the organoids, or rather some parts of them.* They may be specified here: mitochondrium, ribosome, fibril, membrane, Golgi apparatus, chloroplast, vacuole, quantosome, chromosome, lysosome-phagosome, microbodies.

3. Summing up, two *biological elementary units:* the biocomplexes and the bioorganoids are mentioned as contents of the bioplasm. To understand their activities at a real scientific level means to know the mechanism these units use to produce *elementary biofunctions.* This mechanism can be cleared up by investigating the cooperativity of the water content, inorganic atoms and organic molecules in biocomplexes and organoids. Accordingly, investigation of the biological elementary units and their functions seems to be a most urgent task of biology. The fulfilment of that demands collaboration between cytologists, biochemists and biophysicists.

References

De Robertis, E., Nowinski, W. W., Saez, F. A. (1965) Cell Biology, W. B. Sauders Company, Philadelphia and London p. 1, 22.

Eichhorn, G. L. (1973) Inorganic Biochemistry, Elsevier scientific publishing company, Amsterdam, London, New York, p. IX, X.

Engelmann, F. (1878) Arch, ges. Physiol. *7* 167

Ernst, E. (1928) ibid. *220* 672

Ernst, E., Scheffer, L. (1928) ibid. *220* 655

Hazlewood, C. F. (1973) An. New York Academy of Sciences *204* 6—631

Hill, A. V. (1930) Proc. Roy. Soc. B. *106* 445

Ling, G. N. (1972) Hydration of Macromolecules; in Water and Aqueous Solutions, (ed. A. Horne), John Wiley et Sons Inc. p. 663—700

Müller, J. (1837) Handbuch der Physiologie des Menschen. J. Hölscher, Coblenz.

Szent-Györgyi, A. (1957) Bioenergetics. Academic Press Inc. New York

Tigyi, J. (1959) Acta physiol. Acad. Sci. Hung. *16* 93—106

Troshin, A. S. (1956) Проблема клеточиой пронищаемости. Изд. Акад. Наук. СССР. Москва Ленинград

Troshin, A. S. (1966) Problems of Cell Permeability. Pergamon Press, Oxford, London.

**** Ions, wich are "bound" in a more or less dehydrated state, while being liberated during some physiological functions, will be hydrated again. The water molecules, forming these water shells around the ions suffer a volume decrease called electrostriction; this process is accompanied by decrease in entropy, and therefore it should be considered an important bioprocess.

[1] E.g. micromanipulation.

Book Reviews

Biomembranes 8. Edited by L. A. Manson. Plenum Press, New York, 1976.

The 8th volume of the series "Biomembranes" deals with research and results based on the fluid-mosaic model of surface membranes. A major emphasis in this area of research is laid on changes in the membrane structure of viruses and carcinogen-induced tumor cells.

A better insight into the distribution and localization of histocompatibility antigens and a study of the genes wich produce them offer a possibility to minimize risks of transplantation. Investigations about the surface alterations of transformed cells form the theoretical basis of the tremendous efforts directed agains cancer and other malignant diseases.

The first chapter presents data on mapping the H—2 and HLA systems. Ample evidence has been obtained to support the view that single or multiple complexes of glycoproteins more in the plane of the membrane, which can be visualized by fluorescence and electron microscopy.

The properties and antigenic nature as well as the reactivity of these antigens are discussed in the form of a dynamic picture about biological membranes.

In the second chapter a survey of the intracellular localization of the transplantation antigens is given, in case of normal mouse tissues.

The following chapter presents data on occurrence and significance of the cell membrane associated antigens, especially in case of chemical carcinogenesis. The very important field of tumor-associated antigens and their immunobiology is discussed in detail.

The fourth chapter of the volume deals with glycolipids and glycoproteins. From Hakomori's laboratory a methodological paper is included which describes a new method of labelling the carbohydrate portions of membrane-surface-exposed proteins.

The last part of the book review the surface alterations found in virus-induced malignant cells. Here, changes in the cell surface glycolipids, glycoproteins, enzymes and differences in lectin-agglutinability are described.

The authors give a good selection of the recent literature in the various fields. This recent volume of the well-known series "Biomembranes" completes our knowledge on the antigenic nature of membranes and is a useful tool in the hand of both biochemists and immunologists.

JUDITH SZELÉNYI

Neurobiology of Invertebrates (Gastropoda Brain). Edited by J. Salánki. Proceedings of a Symposium held in Tihany, 8—10 September 1975. Akadémiai Kiadó (Publishing House of the Hungarian Academy of Sciences), Budapest, 1976.

This Symposium was the third organized at Tihany in the last years whose major topic was the neurobiology of invertebrates. It seems to have been the most uniform and most comprehensive one of the three. In the 32 lectures nearly all the essential problems of this field were investigated by up-to-date

methods. Probably the volume of this highly succesful conference will become a book often used by many neurobiologists. There were 75 participants, 2/3 of them from 15 foreign countries. The rich material has been divided into 6 thematic units by the Editor helping the reader to an easy survey: (1) Functional identification and mapping in the central nervous system, (2) Monoamine metabolism, uptake and localization, (3) Receptor pharmacology, (4) Electrical properties and mechanism of excitation, (5) Sensory pathways and central projection and (6) Input-output relations and control of behaviour. The printed material amounts to 650 pages at a high level of printing. The illustrations are also of good quality.

The first chapter deals with neuron identification, for which the *Gastropoda* brains offer an excellent experimental material. In a comparative study by D. A. Sakharov useful criteria of nerve cell homologies are formulated. In the comprehensive schemes of I. Kiss and J. Salánki as well as of J. Pusztai and his co-workers not too numerous but rather carefully identified neurons of *Lymnea* and *Helix ganglia* are demonstrated. It seems that the identification of certain neurons of *Gastropoda* brains will be limited by objective factors in addition to the well-known methodological ones. Therefore a strategy based on some well-selected criteria seems to be justified as the occurrence of cells sometimes quite impossible to identify individually may complicate further research. In his concluding remarks G. A. Kerkut witty mentioned that the identifiable cells were the same all over the world.

The neurochemical works presented at the Symposium were concentrated on those monoamines which seem to be or suspected to be neurotransmitters in *Gastropoda* brains. Thus it was mainly the metabolism of dipamine (N.N. Osborne, L. Hiripi), serotonin (C. A. Marsden) and histamine which was investigated by using biochemical anc combined methods (K. Elekes and co-workers). The chapter on Receptor Pharmacology includes two topics. R. J. Walker and his co-workers have studied different amino acid receptors of *Helix aspersa* neurons. All other lectures were dealing with the effects and mechanism of action of certain convulsants and anticonvulsants. This trend of research seems to be very promising for an understanding of such abnormal electrogenesis and contributes to the development of cellular electropathology (M. R. Park, M. R. Klee, J. Hoyer, W. D. Heiss and L. Erdélyi).

The 4th chapter in the volume include 10 papers. In most of them the voltage clamp technique has been applied for studying the biophysics of the membrane of mollusc neurons. P. G. Kostyuk emphasized the peculiarities of the soma membrane. W. Daudnicht preferred an ion-exchange model to interpret his results. M. Gola discussed in an elegant and exhaustive way the electrical properties of bursting pacemaker neurons, both of *Aplysia* and *Helix*. M. Boisson and N. Chalazonitis described interesting effects of exogenous catecholamines and their accumulation. I. Vadász and J. Salánki analysed the mechanism of spike and burst generation in bimodal pacemaker cells. M. Mirolli dealt with passive membrane properties in two lectures. M. Colding-Jørgensen discriminated between two types of adaptation in *Helix* neurons.

In the two last chapters papers on sensory and integrative processes in *Aplysia*, *Lymnea*, *Helix*, *Onchidium* species were presented. Tactile system (C. Janse), extraocular photoreception (C. J. Stol) and chemoreception (J. Salánki and Truong Van Bay as well as B. Jahan-Parwar and S. M. Fredman) were equally investigated in different species. Y. Katayama and K. Murate reported their nice studies on the input-output relationships of identified *Onchidium* neurons (a marine pulmonate). They presented a tentative network consisting of more than ten neurons with probable wiring. They emphasized the small number of detectable direct connections, a major difficulty in network analysis. K. S. -Rózsa as well as H. van Wilgenburg and J. V. Milligan presented results of their detailed studies on the central regulation of cardio-respiratory activity in *Helix pomatia* L. Papers of others (M. Pasic et al., P. M. Balaban, E. G. Litvinov, J. W. Jacklet and J. Rine, T. A. Vlieger and co-workers as well as J. T. Goldschmeding) reported numerous interesting aspects of reflex and motor activities of the mentioned *Gastropods*.

E. LÁBOS

Neurobiology of Invertebrates (Mechanism of Rhythm Regulation). Edited by J. Salánki. Proceedings of a Symposium held in Tihany, 2—5 August, 1971. Akadémiai Kiadó (Publishing House of the Hungarian Academy of Sciences), Budapest, 1973.

The Conference was organized as a Satellite Symposium of the 25th International Congress of Physiological Sciences with 51 participants from 12 different countries. The key words of the papers delivered at the meeting are well expressed by the title. As far as the methods employed, the spectrum of species investigated, the level of experiments presented varies a great deal. Thus, molluscs, insects or crustacean equally occur among the favorized experimental animals. The scope of the lectures is also wide, as papers dealing with single neurons and with the behavioural patterns of animals are equally represented in this volume. The quality of printing including the illustrations is excellent.

The volume consists of four chapters with the following titles: (1) Single Neuron (6 papers, 122 pages), (2) Cardiac Regulation (6 papers, 110 pages), (3) Neuro-Effector System (8 papers, 110 pages), (4) Behaviour and Integration (11 papers, 145 pages). The opening lecture was presented by J. Salánki. He gave account of investigations on rhythm regulations occurring in molluscs both at the level of single neurons and that of motor activity. Most studies in chapter one were carried out with giant neurons of different *Gastropoda* species. D. O. Carpenter's paper discussed ionic mechanisms of endogenous discharges of *Aplysia* neurons. H. Wachter and W. A. Wilson analyzed the generation of slow waves in bursting neurons by the voltage clamp technique. S. N. Ayrapetyan investigated the temperature dependence and sodium pump mechanisms of the rhythmic activities of *Helix neurons*. The pacemaker activity of *Helix neurons* was studied with the voltage clamp technique by E. Neher. Two papers, that of I. Kiss and T. Radil-Weiss et al. dealt with statistical analysis of spontaneous rhythmic activity. Mammalian neurons were investigated only in the paper of T. Radil-Weiss with a comparative purpose. The material presented in this chapter makes a valuable contribution to our knowledge about neuronal rhythmicity.

In the chapter of "Cardiac Regulation" the reader will find accounts of experiments carried out with insect and crustacean preparations as well. Both chemical and neural control were dealt with by the 12 authors. Molluscan preparations were used by M. J. Greenberg's group, K. Kuwasawa and R. B. Hill as well as K. S. Rózsa and I. V. Szőke. M. J. Greenberg and his co-workers studied the effects of extracts of molluscan ganglia on heart preparations. K. Kuwasawa and R. B. Hill touched the problem of ventricular rhythmicity by using various methods. A comparative aspect prevailed in the work of K. S. Rózsa and I. V. Szőke as they used both *Locusta* and *Helix* preparations. In papers by K. Richter and T. Miller the myogenic automatism of the cockroach heart and its innervation were studied. The role of electrogenic Na^+ pump in the modulation of crustacean heart rate was investigated by D. R. Livengood and K. Kusano.

In the third part of the volume 3 papers deal with molluscan preparations, 4 with insects and 1 with a comparison mammalian and *Coelenterata* smooth muscle preparations. D. Willows has analyzed the role of neuronal interactions in the swimming of *Tritonia* (nucibranch). The cardio-respiratory reflexes and the central control of this activity in *Helix pomatia* were investigated by H. Van Wilgenburg. The morphology of the *Anodonta* adductors was examined by I. Zs. Nagy and J. Salánki at different phases of the animal's periodic activity. Investigations on insect preparations cover such subjects as the courtship activity of grasshopper (N. Elsner), patterning of dipterian flight movements (R. J. Wyman) and impulse activity in cockroach (H. Schult and H. Schwarzberg). One of the most exhaustive studies is that of N. Elsner on the courtship of *Gomphocerippus rufus* L.

Among the animals applied by the authors of the last chapter worms and crayfish will be found. J. V. Lary jr. described central pacemaker hierarchies and peripheral feedback in the movement of worm. M. F. Bennett's paper is dealing with circadian rhythms as the main subject evoked great interest. Also circadian rhythms but of

neuronal activity of *Aplysia* were studied by J. W. Jacklet. He estimated the minimum number of neurons involved in circadean oscillations. The visual system responsiveness of crayfish was shown to exhibit diurnal variations (H. Arechiga and co-workers). V. P. Tyshenko also analysed daily rhythms of insect neurons. The role of neurohormones was studied by L. Hiripi and J. Salánki as well as by I. M. Cooke and D. K. Hartline. Spontaneous and evoked rhythm patterns were studied by E. Lábos using the larval form of *Anodonta*. Dishabituation and sensitization in *Aplysia* nervous system was analyzed by T. J. Carew and others. Paper on the tidal activity rhythms of crab (E. Naylor and co-workers) demonstrates the rather wide spectrum of the volume.

E. LÁBOS

Quantum Biochemistry and Specific Interactions by Zeno Simon. Abacus Press, Tundridge Wells, Kent, 1976, 251 pages.

In his book Zeno Simon dealt with the most current theoretical problems of molecular biology. He shows how quantum chemical calculations, very effective in the investigation of small molecules, can be used in the elucidation of the properties of biological macromolecules containing thousands of atoms and discusses which of the classical and semiclassical physico-chemical methods could be suitable for the investigation of the structure and function of proteins and nucleic acids as well as their interactions.

The book consists of seven, more or less independent chapters and six appendices.

In the first, introductory chapter the author discusses the fundamentals of thermodynamics and reaction kinetics. The following chapter describes the traditional quantum chemical calculations concerning molecular orbitals. The third — and longest — chapter contains those semi-empirical formulas, classical and semi-classical physico-chemical relationships which can be successfully used in the description of molecular interactions. In the next four chapters special calculations and results concerning the conformations and interactions of proteins, nucleic acids as well as their thermodynamic parameters are presented.

In view of the fact that quantum biochemistry and the theoretical investigation of macromolecules in general are in the course of very rapid development, this book, first published in 1973 in Rumanian cannot be expected to be up-to-date in every respect. However, it may serve as a sound basis for those who wish to get an inside view of theoretical biochemistry. This is facilitated by several hundreds of carefully selected references.

Zeno Simon's book can be recommended as an introductory reading to those who wish to work in the field of theoretical biochemistry in the future. In addition, however, owing to the numerous data and experimental results it contains as well as to its thorough bibliography it will serve as a handbook for those already working in this field.

I. SIMON

Calcium in Biological Systems. Edited by C. J. Duncan. Symposia of the Society for Experimental Biology. No. 30. Cambridge University Press. Cambridge, 1976. 485 + VIII. pages.

In the last decade, much experience has accumulated pointing to the important role of calcium in a number of cell regulatory processes. Regulation of membrane permeability processes of contraction and secretion, cell division and interaction with cyclic nucleotides are only examples of the biological functions in which calcium plays a central role.

This book is an exciting collection of experimental results recently published in this field. It contains 22 lectures by 46 authors, read at the symposium at Englefield Greend (Surrey, UK), 9 – 12 September 1975.

In the first paper R.J.P. Williams discusses the chemistry of calcium and the kinetics and thermodynamics of calcium binding. This is followed by an excellent article by Mary R. Truter on the chemistry of calcium ionophores, including a detailed description of natural Ca-ionophores (X-537A, A23187,

1-avenaciolide) as well as synthetic products (cyclic depsipetides, monomacrocyclic polyethers, macrobiocyclic compounds). It was discovered in the sixties that the bioluminescent medusa *Aequorea aequorea* contained a special protein, "aequorin", showing luminescence only in the presence of calcium ions. A very sensitive method, based on the above phenomenon and suitable for the detection of as little as $10^{-6}-10^{-7}$ M Ca^{2+} is described in an article by O. Shimomura and F. H. Johnson.

The central regulatory role of calcium is the subject of numerous articles. P. F. Baker investigates the regulation of calcium transport and the triggering effect of calcium on various cell functions in the giant axon of the squid. Mitochondrial calcium permeability is discussed by E. Carafoli and M. Crompton and transcellular calcium transport by A. R. Terepka et al. R. W. Meech gives a detailed account of the interrelationship between the value of membrane potential and the intracellular calcium content. Effects of calcium on cell division and cyclic nucleotides are described by M. J. Berridge.

Relatively much is known about the role of calcium in muscle contraction and its binding to muscle proteins. These problems are covered by several articles of this book (J. H. Collins, A. G. Szent-Györgyi, S. R. Taylor and R. E. Godt, S. Ebashi et al., R. Niedergerke et al. and C. C. Ashley et al.).

It is, of course, impossible to give an account of all the articles of this volume in such a short recension. In the last article A. Weber brilliantly evaluates the individual lectures of the symposium and the progress made in the various fields.

To sum up this volume gives an excellent review of the physiological and biochemical research activities concerning the bioregulatory role of calcium.

The book will certainly be very useful for scientists working in this special field as well as for physicians and biologists who just would like to follow tenit trend of research.

G. GÁRDOS

New methods for the analysis of coagulation using chromogenic substrates. Edited by I. Witt. Proceedings of the Symposium of the Deutsche Gesellschaft für klinische Chemie. Titisee, Breisgau. West-Germany, 1976. Walter de Gruyter. Berlin—New—York 1977.

The book (275 pages and 41 figures) presents the lectures and discussions of the conference in Titisee on new methods of coagulation analysis using chromogenic substrates. Twenty selected papers are divided in four main chapters, the *first* of which deals with the theory of coagulation analysis with chromogenic substrates. (Molecular aspects of fibrinogen-fibrin-transition. Determination of protease activities with specifically constructed peptide-p-nitroanalide derivatives. pp. 1—54.) *Chapter 2* entitled "Determination of thrombin, antithrombin and antifactor Xa" consists of seven articles as follows: Comparison of six different methods for the quantitative assay of antithrombin III. Assay of antithrombin III with the chromogenic substrate S—2160. Effects of thrombin and low heparin concentration on the antithrombin activity in human plasma. Enzymatic determination of thrombin and thrombin inhibitors. Studies on the determination of antithrombin. Studies on antithrombin III using Chromozym TH. The determination of antithrombin III and antifactor Xa activity. (pp. 55—142.) The *3rd chapter* gives detailed informations on the determination of prothrombin.) Set—— on the determination of prothrombin. (Determination of plasma prothrombin with the chromogenic peptide substrate H-D-Phe-Pip-Arg-pNA (S—2238). Determination of plasma prothrombin with Chromozym TH. Studies on the determination of prothrombin with S—2160. Amidolytic assay of prothrombin activated with Ecarin, a procoagulant from *Echis Carinatus* venom.) (pp. 143—180.) *Chapter 4* contains several methods for the determination of various coagulation factors. (Assay of factor Xa with a chromogenic substrate. A new assay for plasma kallikrein activity utilizing a synthetic chromogenic substrate. Heparin assay in plasma. Antiplasmin determination by means of the plasmin specific substrate S—2251; methodological studies and some clinical applications. The quantitative determination of urokinase with Chromozym UK. Estimation of urokinase activity by means of a highly susceptible synthetic

chromogenic peptide substrate.) (pp. 181—262.) *Chapter 5* summarizes the general closing discussion including important proposals (e.g. the organization of task forces for the standardization of the reactins carried out with chromogenic substrates), too.

It's always very useful to get summarizing information about new analytical methods, especially if they represent a new trend. The recent progress in the development of synthetic chromogenic substrates is clearly demonstrated by this book. The use of these compounds in the analysis of coagulation is of special interest both from theoretical and practical point of view.

It may be possible that the time comes when chromogenic substrates will widely be used in laboratories. This hope is supported by some common advantages of the various assay methods so far elaborated. However, at present it would not be correct to suppress some problems yet unsolved. First of all the specificity of some chromogenic substrate proved to be insufficient in comparison with that of the natural one. (e. g. Problems in the assay of thrombin using synthetic peptide as substrate — in Thrombosis Research *10*, 549—56, 1977.) Discussions presented in the book reflect many other problems in this relation, too. As Prof. L. Roka said in his appropriate closing remark: "...man muß nun herausfinden, welche Substrate zu welchen Enzymen passen" (one must only find out, what kind of substrates fit what enzymes).

The book is warmly recommended to those interested in the analysis of blood coagulation.

D. BAGDY

Kahrig, E., Besserdich, H.: *Dissipative Strukturen.* Fortschritte der experimentellen und theoretischen Biophysik, Band 21. VEB Georg Thieme Verlag, Leipzig, 1977, 166 p., (in German).

As it is known, the decisive impetus to what is now called the field of dissipative structures was given by the elaboration of the comprehensive framework of non-linear thermodynamics for systems far from equilibrium. This important achievement is mainly due to the pioneer activity of the Brussels group that formed around Prigogine. A fundamental contribution to this new scientific branch, that not only lies between physics, chemistry and biology but also includes comparatively independent regions of these disciplines, was unquestionably the book entitled "Thermodynamic theory of structure, stability and fluctuations" by Glansdorff and Prigogine. Since the publication of this book in 1971, a number of monographies have appeared; the two latest one year before and one year later than the book under consideration.

It was, therefore, not very easy for the authors to make a contribution that is not simply the i+1-th in the series but offers something new and valuable either in the choice of the subject matter or in the way of the presentation. It is to be stated that the authors have succeeded in doing so and produced a well written contribution of remarkable conciseness and clearness which cannot be overappreciated.

The task was not easy, all the more so, as the field is rather multidisciplinary than interdisciplinary in character and this is not merely a different expression of synonyms, for it requires a multidisciplinary treatment as well. On the other hand, according to the editing policy of the series which aims to provide the reader with easily readable texts of introductory character, the authors were naturally not in a position to follow consequently the multidisciplinary lines of treatment and to go into detail on the particular domains concerned. As a result of this restriction they had to create an original classification of dissipative processes that do, or may, play a role in the formation and maintaining of a particular dissipative structure.

According to this ordering principle, the book is divided, – apart from a short introductory chapter on the theoretical vackground mainly considering stability conditions in non equilibrium systems, – into chapters dealing with dissipative structures in (i) physical, (ii) chemical, and (iii) biological systems. In addition, a final chapter of a few pages is included in which J. Erpenbeck, from the Institute of Philosophy, GDR Academy of Sciences, gives a philosophical account of the topic, the evaluation of which is beyond the scope of this recension.

The chapter on dissipative structures in physical systems starts, evidently, with the Bénard phenomenon. Here the way of treatment becomes particularly advantageous since, as it is well known, the formation of cellular-like structures in liquid layers spread over solid substrates due to a unidirectional temperature gradient which was first considered to be a result of buoyancy and heat conduction effects only, and the essential role of surface tension was recognised but fifty years later. Chapter 3 also includes a very valuable and comprehensive account on dissipative structures as connected with the dynamics of fluid interfaces written by Professor Linde, Institute of Physical Chemistry of the GDR Academy of Sciences, who is a well-known expert of this particular field.

Dissipative structures and chemistry, being the subject matter of Chapter 4, are treated, not surprisingly, from the point of view of chemical reactions that oscillate either in time or in space or both. The interest is focused on the Zhabotinsky reaction but some other types, such as the classical Liesegang ring formation are also dealt with to some extent.

Biology occupies twice as much space as chemistry does but even so Chapter 5 consists of no more than 20 pages about the half of which considers biochemical oscillations at the metabolic, epigenetic and cellular levels, respectively. The rest of this chapter is divided into sub-chapters on modelling of biological transport-reaction systems, morphogenesis, and mechanochemical effects.

The book is richly illustrated (61 figures), the subject matter well arranged, and the literature references are up-to-date. Also the price is reasonable, so that the contribution of Kahrig and Besserdich can be recommended to all who are interested in getting a general survey of the field.

Having read this book, the recensent feels entitled to make a remark on how specialisation, even if resulting in the integration of disciplines using seemingly different approaches might lead to disregarding well-known experimental facts as well as basic principles of other disciplines. It is, for instance, well known that colloids and other supermolecular systems are inexhaustible examples for the formation of thermodynamically non-stable material structures in non-living systems. Yet, the importance of colloids for the further understanding of dissipative structures appears not to be taken into account to such an extent as it should be. This remark is, however, not a critic on this book but rather a suggestion for those who have become interested in the topic at hand and intend to broaden our knowledge on dissipative structures on an experimental rather than purely a theoretical basis. It is believed that the very link between chemistry and biology, besides biochemistry of course, is undoubtedly colloid science. This has been repeatedly emphasized for many years by such outstanding scientists as Eric Rideal in Cambridge, Aladár Buzágh in Budapest and Piotr Rehbinder in Moscow but, unfortunately, has not been generally recognized. But facts remain facts, and it cannot be disregarded that the structural background, we may say the stage, on which the whole complex of pehomena, called life, manifests itself is so intimately connected with colloid systems and surface phenomena that any destruction of the structural organisation of this kind results in what is called death. So it is time for colloid science to be considered as a discipline which might very effectively stimulate further development of both biology as a whole and biophysics in particular.

E. WOLFRAM

Biomorphose von Zellorganellen und Methoden ihrer submikroskopischen Untersuchung. — Ergebnisse der 8. Arbeitstagung "Elektronmikroskopie" (Programmabschnitt Biologie und Medizin) der Gesellschaft für Topochemie und Elektronmikroskopie der DDR und des Fachverbandes Elektronenmikroskopie der Physikalischen Gesellschaft der DDR in Berlin vom 27. bis 30. Januar 1975. — Acta histochemica, VEB Gustav Fischer Verlag, Jena, 1976. (in German)

The volume consisting of 390 pages contains the material of the 48 lectures performed at the conference. The lectures dealt with two subjects: 1. Biomorphosis of cell organelles (30 lectures); 2. Methods of research (18 lectures).

The lectures on biomorphosis of cell organelles embrace the newer results obtained in analyses of function of cytoplasmic organelles in different cells. The most important topics of this chapter are: the structure and genesis of endoplasmic reticulum, the subcellular organization of protein biosynthesis, inner topography of mitochondria, enzyme hystochemistry, lysosome-investigations in different functional conditions, investigations of cell-membrane, macromolecular organization of plastides and chloroplastides, independent examinations of certain types of cells, etc.

In the lectures dealing with the experimental methods the solution of certain experimental problems can be found beside the presentation of the method. The following methods are discussed: immunelectronmicroscopy, possibilities of the application of cryoultramicrotome in membrane experiments, the application of autoradiography in detection of soluble compounds, enzymes, combination of X-ray microanalysis and electronhistochemistry, etc.

201 figures of good quality and 21 tables illustrate the material of lectures and each lecture is supplemented with detailed list of references.

After the lecture presentations, in a short chapter two reviews give summarizing evaluation of the round-table programme of the conference.

The functional-morphological approach of the themes is characteristic to the lectures presented at the conference. The methods embrace several alternatives of modern electronmicroscopic procedures. The nicely got-up book affords valuable informations to the research-workers interested in the biomorphosis of cell-organelles and the functional morphology of the cell.

L. KOMÁROMY

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The XIth International Congress of Biochemistry will be held in Toronto, Canada, July 8–14, 1979. It is being hosted by the Canadian Biochemical Society and sponsored by the International Union of Biochemistry.

The scientific program will reflect the current state of the most rapidly developing fields of biochemistry in 1979. Papers will be presented at symposium sessions by invited speakers. Submitted papers will be accepted for presentation in the form of posters. Symposia and poster sessions will be devoted to each of the following 13 topics.

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