VOLUME 15
NUMBER 1
1980

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

ABBPAP 15 (1) 1–79 (1980)

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

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Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Volume 15

Akadémiai Kiadó, Budapest
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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 1–4 (1980)

Quantitative Evaluation of Ion-Exchange Thin-Layer Chromatograms by Video-Densitometry (IV.) Determination of Diaminopimelic Acid

(Short Communication)

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(Received October 20, 1979)

Diaminopimelic acid – a constituent of the cell wall mucopeptide of bacteria – is considered as a suitable marker of microbial protein synthesis in the alimentary canal of ruminants (Krawielitzki, Piatkowski, 1977). Its determination is usually carried out by subjecting rumen digests to acid hydrolysis and subsequently analyzing them by ion-exchange column chromatography (Krawielitzki, Piatkowski, 1977; Hutton et al., 1971.) or gas-liquid chromatography (Sen et al., 1969). Colorimetric (Work, 1957) and spectrofluorimetric (Rogers et al., 1967) methods were also developed; however, the use of the latter methods is limited by

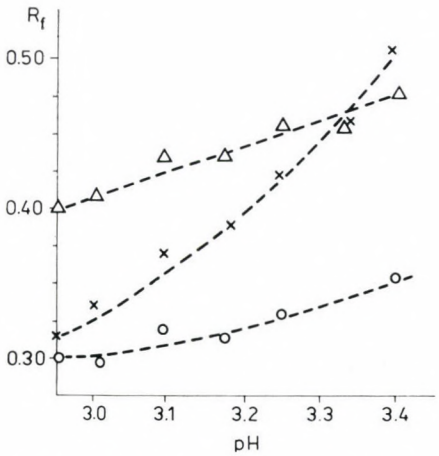

Fig. 1. Effect of the pH of the eluting buffer on the separation of diaminopimelic acid. Eluting buffer: 0.4 M sodium citrate. +–+: DAPA; △–△: Val; ○–○: Met

the interference of the protein-constituting amino acids present usually in large excess as compared to DAPA.

In the present paper a method is described for the separation of DAPA by ion-exchange TLC on resin-coated chromatosheets (Dévényi et al., 1971). Com-

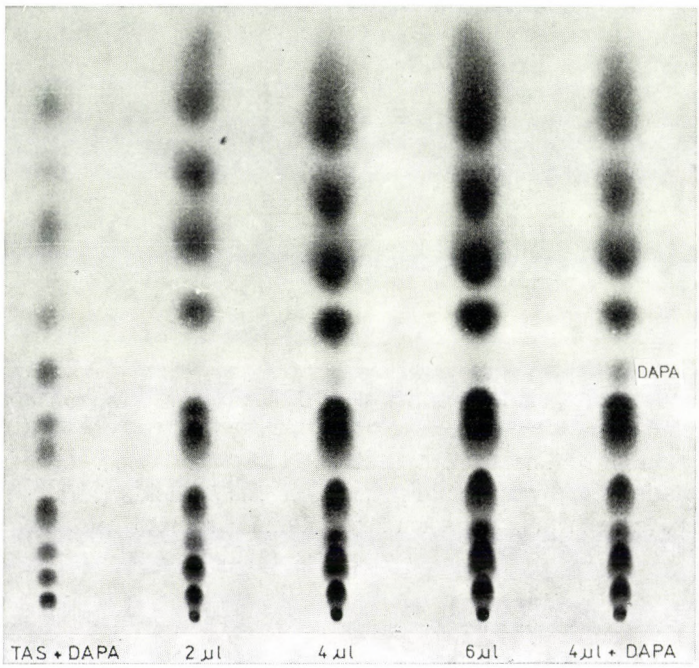

Fig. 2. Separation of diaminopimelic acid on an ion-exchange chromatosheet. Eluting buffer: Sodium citrate, pH 3.14 ($Na^+ = 0.4$ M). Samples: 1 Amino acid calibration mixture, DAPA added; *2—4* rumen digest hydrolysate 2,4,6 µl, respectively. For sample preparation see text

bined with video-densitometric quantitation (Dévényi, 1976), this procedure provides a simple means for determining DAPA in biological samples.

Ion-exchange TLC sheets (Fixion 50 × 8, Chinoin, Hungary or Ionex 25 SA-NA, Macherey, Nagel + Co, Düren, GFR) allow the separation of the protein-constituting amino acids by a single chromatographic run using sodium citrate buffer, pH 3.3, ($Na^+ = 0.4$ M) for development. However, DAPA is not separated from valine under these conditions. By varying the pH of the buffer in the range of 2.9—3.5 it was found that the R_f of DAPA depends on pH to a greater extent than that of the adjacent amino acids, valine and methionine (Fig. 1). This provides a

Abbreviations: DAPA, diaminopimelic acid; TLC, thin-layer chromatography.

possibility to achieve good separations at pH 3.14 ($Na^+ = 0.4$ M). The composition of the buffer is as follows:

Citric acid · H_2O	84 g
Sodium hydroxide	16 g
Hydrochloric acid, 37%	8 ml

made up to 1000 ml with deionized water; pH adjusted to 3.14 at 25 °C by dropwise addition of concentrated HCl.

The chromatosheets were equilibrated by continuous development for 24 h with the eluting buffer diluted 10-fold.

Chromatography and staining with Cd-ninhydrin was carried out as described by Dévényi (1976). A typical chromatogram is shown in Fig. 2. The color of the DAPA spot is purple turning to brownish on standing at room temperature for 2–3 days. Quantitative evaluation was carried out with a Telechrom S type video-densitometer (Kerényi et al., 1980) in transmission mode after ovenight color development. In the range of 0.5–6.0 μg DAPA a linear calibration curve was obtained (Fig. 3).

On the basis of the above findings the following conditions were chosen for sample preparation:

Rumen contents (taken from fistulated Hungarian Merino wethers) were strained through cheese cloth. The liquor was allowed to sediment for 10 minutes and the supernatant was centrifuged at 7000 *g* for 10 minutes. 0.5 g of the wet pellet

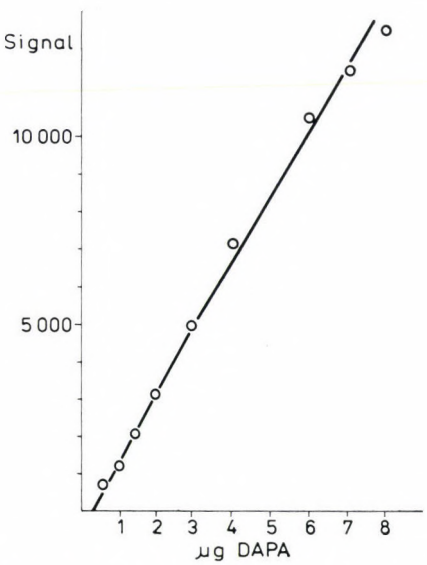

Fig. 3. Calibration curve of diaminopimelic acid determined by the use of a Telechrom S video-densito-meter

was weighed in a hydrolysis vessel, 2.0 ml of 6 N HCl and 0.5 ml of concentrated HCl were added (the latter compensates dilution caused by the samples' water content). Hydrolysis was carried out at 105 °C for 48 h. The hydrolysate was filtered through filter paper which was subsequently rinsed with 2 ml distilled water. The combined filtrate was evaporated to dryness. The dry residue was redissolved in 0.2 ml distilled water. 3–6 μl of this solution (corresponding to about 1–4 μg of DAPA) was spotted onto the ion-exchange chromatosheet. The reproducibility of the procedure (hydrolysis, chromatography and quantitation) is characterized by a coefficient of variation of 6% (determined from 12 parallel measurements). The recovery of DAPA, added to the samples prior to hydrolysis, was found to be 90%, in good agreement with the data reported by Hutton et al. (1971).

The present method provides a simple and convenient tool for studying the fate of microbial cell walls in the alimentary canal of ruminants. The ion-exchange TLC procedure described here separates DAPA from the large excess of common amino acids in a single step, which is not achieved by any of the column chromatographic methods reported so far.

The authors wish to thank Dr T. Dévényi for his help in developing the method. Thanks are due to Dr M. Sajgó for helpful discussions.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 5–19 (1980)

Fluorescence Energy Transfer Studies on Normal and Leukemic Mouse Lymphocytes

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(Received July 12, 1979)

Control and acute leukemic cells of AKR : Lac C3H mg : Lac/F_1 hybride mice were labeled either with ethidium bromide or ethidium bromide plus fluorescamine. Theoretical and experimental evidence shows that the ethidium bromide binding ability of cells and the average efficiency of Förster-type fluorescence energy transfer between fluorescamine and ethidium bromide inside the cells depend on both the cell and dye concentrations. It is shown further that fluorescamine treatment enhances the uptake of ethidium bromide by both normal and leukemic cells.

A sevenfold difference was found in ethidium bromide binding ability between normal and leukemic cells at optimal dye concentration.

Introduction

The advent of flow microfluorimetry and fluorescence cell sorting techniques provided an excellent tool to study large cell populations in suspension without losing information about individual cells (Arndt-Jovin, Jovin, 1974; 1977). Fluorescence labeling is an obligatory requisite of such techniques since the labels provide information about the characteristics of different cell types. In many cases double labeling is necessary in order to provide an extension of the parameters e.g. for cell separation (Crissman, Steinkamp, 1973; Hawkes, Bartholomew, 1977; Steinkamp et al., 1974; Stöhr et al., 1977).

The present paper deals with the fluorescence labeling of lymphoid cells from AKR hybrid mice either with ethidium bromide or ethidium bromide plus fluorescamine. The two dyes were used as a donor-acceptor pair to elicit the Förster-type singlet-singlet resonance energy transfer process to study the structural differences between control and leukemic lymphocytes. Since both ethidium bromide and

Abbreviations: EB, ethidium bromide; [E], ethidium bromide concentration; PBS, phosphate buffered saline; DMSO, dimethylsulfoxide; N, intercalation sites of ethidium bromide in chromatin; X, intercalated ethidium bromide; C, cell concentration; F, fluorescence intensity.

Footnote: According to the uncorrected spectra of bound dyes, the wavelengths of maximal excitation and emission were found to be 385 nm and 470 nm for the donor (fluorescamine) and 520 and 600 nm for the acceptor (ethidium bromide), respectively.

fluorescamine have multiple targets inside the cell to bind to, a thorough binding analysis is required before conclusions can be drawn from the results. A theoretical model of binding kinetics has been developed to facilitate evaluation of experimental data gained by the above double labeling technique. The results of the analysis facilitate the application of the fluorescamine-ethidium bromide donor-acceptor pair for studying the chromatin of cells in vivo.

Materials and methods

Cells. Cells of control and acute lymphoid leukemic AKR : Lac C3H mg : Lac/F_1 hybride mice were obtained from the lymphnodes. The animals were anaesthetized with ether, and the mesentheric and paraortic lymphnodes of 10 animals were combined, cut into small pieces, suspended in PBS, pH 7.2, and filtered through gauze. Cell number of the filtered solution was determined by conventional counting and adjusted by dilution with PBS to 1×10^7 cell/ml. Leukemic cells were produced by injecting abdominally: 0.1 ml of a cell suspension (1×10^6 cells/ml) from the spleen of spontaneous leukemic AKR : Lac mice into hybrid mice. The animals showed the classic picture of acute lymphoid leukemia at day 10 – 12 and died after an additional 2 – 4 days. Animals from this period were used to gain leukemic cells. Owing to the size of these lymphnodes one leukemic animal could provide the necessary amount of cells. The methods of preparation was the the same as in the case of control cells.

Fluorescence labeling. The cells were labeled with fluorescamine dissolved in dimethylsulfoxide (DMSO), spectroscopic grade from Merck, in the following way: To 1.9 ml of a cell suspension containing 10^7 cell/ml, taken from the stock solution thermostated at 37 °C, 0.1 ml of fluorescamine in DMSO was added. The mixture was vigorously shaken for about one minute. The three different fluorescamine concentrations used during the labeling were 0.14 mg/ml, 0.07 mg/ml and 0.035 mg/ml. Since the fluorescamine formed a covalent adduct with the primary amines of the cells, further dilution of the labeled cells did not alter the amount of the dye in a single cell (Damjanovich et al., 1977).

For EB treatment, the given amount of the above, fluorescamine labeled cell suspension was mixed with PBS and 0.05 ml of EB in such a way that the total amount of the new suspension was always 0.7 ml while cell concentration varied from sample to sample. The final EB concentrations were 1.85×10^{-5} M, $9.06 \times$ $\times 10^{-6}$ M and 4.53×10^{-6} M, respectively. These 0.7 ml samples were used for fluorescence measurements. When only EB labeling was carried out the only deviation from the above described treatment was the use of DMSO alone instead of fluorescamine in DMSO.

Measurement. A Hitachi MPF-4 spectrofluorimeter was used for steady state measurements. The suspensions were pipetted into a 0.3 ml silica cell and the intensity measurements were carried out at 25 °C.

Results

Theoretical

Ethidium bromide binding. Ethidium bromide is one of the well-known intercalating dyes introduced by LePecq and Paoletti (LePecq, Paoletti, 1967). Although EB can bind to nucleic acids, or even to proteins in several ways (Krakow et al., 1968), the most important way of binding is intercalation. The planar dye intercalates into the major grooves of the double helical nucleic acids. This type of binding prevents a proton transfer of the excited forms of the dye to water and thus increases the fluorescence quantum yield by a factor of nearly twenty (Bontemps, Fredericq, 1974; Olmsted, Kearns, 1977).

This property of EB makes it possible to investigate a few cellular parameters through the kinetics of EB intercalation.

Studying these kinetics in the case of an *in vivo* cell population, one has to assume that the intercalation of the dye can be characterized by a set of association constants K_i ($i = 1, 2, \ldots, n$), each of them belonging to a given double helical structure of nucleic acids. On the basis of experiments on EB intercalation into different types of double stranded nucleic acids (Aktipis, Kindelis, 1973; Angerer, Moudrianakis, 1973; Bontemps, Fredericq, 1974; Genest, Tichadou, 1977; Jovin, Striker, 1977; Plumbridge, Brown, 1977; Tritton, Mohr, 1973; Waring, 1965) one can assume that in the case of living cells the K_i values range roughly from $10^3\ M^{-1}$ to $10^6\ M^{-1}$.

In a general case there is a set of n reaction schemes for intercalation:

$$E + N_i \underset{k_{-i}}{\overset{k_i}{\rightleftarrows}} X_i\,; \quad K_i = \frac{k_i}{k_{-i}} \qquad (i = 1, 2, \ldots, n) \tag{1}$$

where E means EB, N_i is the *ith* type of free intercalation sites characterized by the association constant K_i, and X_i is the EB being intercalated into the *ith* type of sites. Assuming that

$$\frac{d[X_i]}{dt} = 0, \text{ and } [N_i] \gg [X_i] \text{ for every } i\,, \tag{2}$$

we have

$$[X_i] = [E_0] \frac{K_i[N_i]}{1 + \sum_{i=1}^{n} K_i[N_i]} \tag{3}$$

and

$$[E] = [E_0] \frac{1}{1 + \sum_{i=1}^{n} K_i[N_i]} \tag{4}$$

where $[E_0]$ is the total EB concentration.

In vitro experimental data show that the quantum yields of EB molecules intercalated into different forms of double stranded nucleic acids are within a narrow range (Olmsted, Kearns, 1977). Therefore, we can use a common quantum yield Φ for all kinds of intercalated EB. Then, for fluorescence intensity F

$$F \propto [E]\varepsilon_0\Phi_0 + \sum_{i=1}^{n} [X_i]\varepsilon\Phi \tag{5}$$

where Φ_0 is the quantum yield and ε_0 is the molar decadic extinction coefficient at the excitation wavelength for free EB molecules, while Φ and ε are the same for the bound ones. When there is no cell in the sample,

$$F_0 \propto [E_0]\varepsilon_0\Phi_0 \tag{6}$$

Instead of the set of $[N_i]$, cell concentration C can be introduced by using α and ξ_i as

$$[N_i] = \alpha C \xi_i ; \quad \sum_{i=1}^{n} \xi_i = 1 \tag{7}$$

where α is the total number of intercalation sites inside a single cell and ξ_i is the fraction of the *ith* type of sites.

It has to be noted that cell concentration C is generally expressed as cell/ml. Therefore, the dimension of $[N_i]$ in the above equation is also ml^{-1}. Thus, for practical use, the value of $F_0/(F - F_0)$ can be written as

$$\frac{F_0}{F - F_0} = \frac{1}{\eta} \frac{1}{\sum_{i=1}^{n} K_i \xi_i} \frac{1}{\alpha C} + \frac{1}{\eta} ; \eta = \frac{\varepsilon\Phi}{\varepsilon_0\Phi_0} - 1 \tag{8}$$

Plotting the values of $F_0/(F - F_0)$ against 1/C, a straight line can be obtained with two intercepts p and q:

$$p = \frac{1}{\eta} \tag{9}$$

$$q = \alpha \sum_{i=1}^{n} K_i \xi_i \tag{10}$$

As a consequence of the ml^{-1} unit used as a measure of cell concentration, the value of q should be measured in ml. (It is obvious that the unit ml can easily be converted into the unit of M^{-1} multiplying it by a factor of 6×10^{20}.) When $n = 2$ and $\Phi_1 \neq \phi_2$, the values of ξ_1, ξ_2 and α become determinable from p and q. In this case η also contains both Φ_1 and Φ_2.

Double labeling. More information can be gained about the characteristics of nucleic acids and also proteins, even inside the chromatin, if a suitable fluorescence

donor-acceptor pair is applied. To get a deeper insight into chromatin structure through the study of Förster-type energy transfer processes, a theoretical treatment of the system seems to be necessary.

During this treatment we restrict ourselves to the case in which the concentrations of donor and acceptor molecules inside the cells are relatively low, i.e. the probability that two acceptor molecules can be found around a donor molecule within the R = 60–100 Å limiting distance of Förster-type energy transfer (Stryer, Haugland, 1967), can be neglected. In order to calculate the donor-acceptor pair concentration and the average transfer efficiency, we divide the donor molecules into m groups according to the distribution of the bound acceptor molecules around them (the energy transfer to free acceptor molecules is disregarded because of the low acceptor concentration).

Then these subgroups can be characterized by a set of $\zeta_j(\phi, \sigma, r)$ (j = = 1, 2, . . . , m) functions in the following way:
Each donor molecule belonging to the *jth* group has a

$$b_j = [X_T] \iiint_{V(R)} \zeta(\phi, \sigma, r)\, d\phi\, d\sigma\, dr = [X_T]\, V_j \tag{11}$$

number of acceptor molecules within the volume V(R). V(R) is a sphere around a donor molecule and has a radius of R. It contains a number of acceptor molecules defined above as b_j. $[X_T] = \frac{1}{vC} \sum_{i=1}^{n} [X_i]$ is the total concentration of bound acceptor molecules inside a cell. Here v is the average volume of a cell and C is the cell concentration as before. The integral over V(R) gives reduced volume. Its value depends on the character of the density function $\zeta(\phi, \sigma, r)$. It is obvious that b_j remains in linear relationship with $[X_T]$ until any kind of binding site (N_i) within V(R) becomes saturated or will be close to saturation. That means that as long as $[X_T]$ is in linear relationship with the total EB concentration, $\zeta_j(\phi, \sigma, r)$ remains unchanged.

The value of the integral in Eq.(11) equals V(R) if the acceptor is uniformly distributed. Otherwise it can be smaller or higher or, perhaps for special groups, the same as the value of V(R) depending on the distribution of binding sites for the acceptors.

Denoting the concentration of donor molecules belonging to the subgroup j by C_{Dj}, the total concentration of effective donor-acceptor pairs C_{DA} is:

$$D_{DA} = \sum_{j=1}^{m} C_{Dj}\, b_j = [X_T] \sum_{j=1}^{m} C_{Dj}\, V_j \tag{12}$$

Assuming that the values of C_{Dj} are well below the saturation value for every j,

$$\frac{C_{Dj}}{C_D} = q_j \tag{13}$$

where C_D is the total donor concentration in the measuring volume. Thus, Eq.(12) can be written as:

$$C_{DA} = C_D[X_T] \sum_{j=1}^{m} q_j V_j \tag{14}$$

It is obvious that the average transfer efficiency, E_j, for the donor-acceptor pairs of *jth* type is:

$$E_j = \frac{\iiint_{V(R)} \zeta_j(\phi, \sigma, r)\, E(r)\, d\phi\, d\sigma\, dr}{\iiint_{V(R)} \zeta_j(\phi, \sigma, r)\, d\phi d\sigma\, dr} \tag{15}$$

where E(r) is the transfer efficiency. E(r) depends on the actual donor-acceptor distance r, and r_0, i.e. the Förster distance, where the probability of the emission by the donor or acceptor is equal (Förster, 1951):

$$E(r) = \frac{r_0^6}{r^6 + r_0^6} \tag{16}$$

Now, having the above relationships, we can describe the fluorescence intensities for the cases in which only acceptor or acceptor plus donor molecules are present in the sample.

In the following the fluorescence intensities are supposed to be determined, unless stated otherwise, by exciting the samples at the excitation maximum of the donor and measuring the emissions at the emission maximum of the acceptor.

When only acceptor molecules are present, one can write for the fluorescence intensity, F_A^0, as in Eq.(5):

$$F_A^0 \propto [E]\, \varepsilon_0 \Phi_0 + \sum_{i=1}^{n} [X_i]\, \varepsilon \Phi$$

Using the above relationships, the intensity F_A^D, when both donor and acceptor molecules are present in the sample:

$$F_A^D \propto [E]\, \varepsilon_0 \Phi_0 + \sum_{i=1}^{n} [X_i]\, \varepsilon \Phi + [X_T]\, C_D\, \varepsilon_D\, \phi \sum_{j=1}^{m} q_j V_j E_j \tag{17}$$

where ε_D is the molar decadic extinction coefficient for the donor at the excitation wavelength. This proportionality can be rewritten into a simpler form:

$$F_A^D \propto [E]\varepsilon_0 \Phi_0 + \sum_{i=1}^{n} [X_i]\, \varepsilon \Phi + [X_T]\, \varepsilon_D \Phi\, C_D \bar{E} \tag{18}$$

where

$$\bar{E} = \sum_{j=1}^{m} q_j V_j E_j \tag{19}$$

The equation

$$\frac{F_A^D}{F_A^0} = 1 + \frac{\varepsilon_D}{\varepsilon} E \tag{20}$$

is the most generally used one for calculation of the transfer efficiency (E). F_A^0, F_A^D, ε_D and ε have the meaning as above. This equation holds only for the special case in which one donor and one acceptor have to be taken into account. From equations (5) and (18) a new form of Eq.(20) can be developed:

$$\left(\frac{F_A^D}{F_A^0} - 1\right)^{-1} = \frac{\sum_{i=1}^{n} [X_i]}{\varepsilon_D C_D \bar{E} [X_T]} + \frac{\varepsilon_0 \Phi_0 [E]}{C_D \varepsilon_D \Phi \bar{E} [X_T]} \tag{21}$$

By the use of Eqs (3), (4), (7), (10) and the definition of $[X_T]$ Eq.(21) can be rewritten as

$$\left(\frac{F_A^D}{F_A^0} - 1\right)^{-1} = \frac{\varepsilon v}{\varepsilon_D \delta \bar{E}} + \frac{1}{C} \frac{\varepsilon_0 \Phi_0 v}{\varepsilon_D \Phi \delta q \bar{E}} \tag{22}$$

where

$$\delta = \frac{C_D}{C} \tag{23}$$

is the average number of donor molecules bound per cell. Even if the cell concentration changes after labeling with fluorescamine, δ remains constant. This comes from the unique properties of the dye, forming a covalently bound fluorescent adduct with primary amino groups within a very short time. The donor (bound fluorescamine) concentration in a single cell:

$$C_f = \frac{\delta}{v} \tag{24}$$

The introduction of C_f makes Eq.(22) more convenient, since C_f is constant after the reaction has been completed, and depends almost linearly on the applied fluorescamine concentration.

Plotting $\left(\frac{F_A^D}{F_A^0} - 1\right)^{-1}$ against $\frac{1}{C}$ the function is a straight line giving two intercepts with the axes:

$$t = \frac{\varepsilon}{\varepsilon_D} \frac{1}{C_f \bar{E}} \tag{25}$$

and

$$s = q(1 + \eta). \tag{26}$$

η, q, $\bar{E}$ and C_f are defined as in Eq.s (8), (10), (19) and (24), respectively.

It is important to note that here $\bar{E}$, in contrast with its usual definition, has a dimension of volume. This is only of practical use and means that the parameter $\bar{E}$ which is in a close relationship with the average transfer efficiency, is not a real efficiency. It also contains other parameters characteristic of the system. However, as the main aim of this study is to compare systems having different structures it seems to be practical to use $\bar{E}$ as a single phenomenological parameter and call it average transfer efficiency.

The theoretical treatment applied above has resulted in an equation that predicts a strong dependence of the transfer efficiency on the applied concentration of the dyes.

Experimental

The principal aim of our experiments was to detect differences in chromatin structure between normal and leukemic cells in vivo, due to an enhanced chromatin activity in the latter. Ethidium bromide alone and together with fluorescamine was applied to reveal structural changes inside the chromatin. In addition, the experiments served to test the validity of the theoretical results outlined in the previous chapter.

EB binding kinetics: In these experiments EB binding was studied using a 520 nm excitation wavelength and monitoring the fluorescence intensity at 600 nm. According to the theoretical treatment the cell concentration was varied in each experiment, while the EB and the fluorescamine concentrations were kept constant inside the cells. Here the application of fluorescamine prior to the administration of EB was introduced only to investigate the influence of the presence of fluorescamine on EB uptake.

The fluorescamine treatment affected the EB binding ability of cells (curves a, b). According to Eq.(8), a double reciprocal plot can be used to linearize the curves

Table 1

Ethidium bromide binding ability

The values of intercept q (in units of 10^{-6} ml), of normal and leukemic cells at different concentrations

		Final ethidium bromide concentration in the samples					
		1.85×10^{-5} M		9.06×10^{-6} M		4.53×10^{-6} M	
Cell type		normal	leukemic	normal	leukemic	normal	leukemic
Fluorescamine concentration during the labeling	0.5 mM	0.11	0.24	0.24	0.20	0.26	0.24
	0.25 mM	0.15	0.20	0.31	0.46	0.31	0.54
	0.125 mM	0.13	0.26	0.22	0.22	0.18	0.22
	none	0.11	0.07	0.06	0.07	0.07	0.07

shown in Fig. 1A. In this way the values of intercepts p and q can be calculated. The linearized form of curves a and b of Fig. 1A can be seen in Fig. 1B.

In order to find differences between normal and leukemic cells, both fluorescamine and EB concentrations were varied in the following series of experiments. Whenever the cell concentration and thus the bound fluorescamine concentration were varied, the concentration of EB was kept constant. For all these cases the

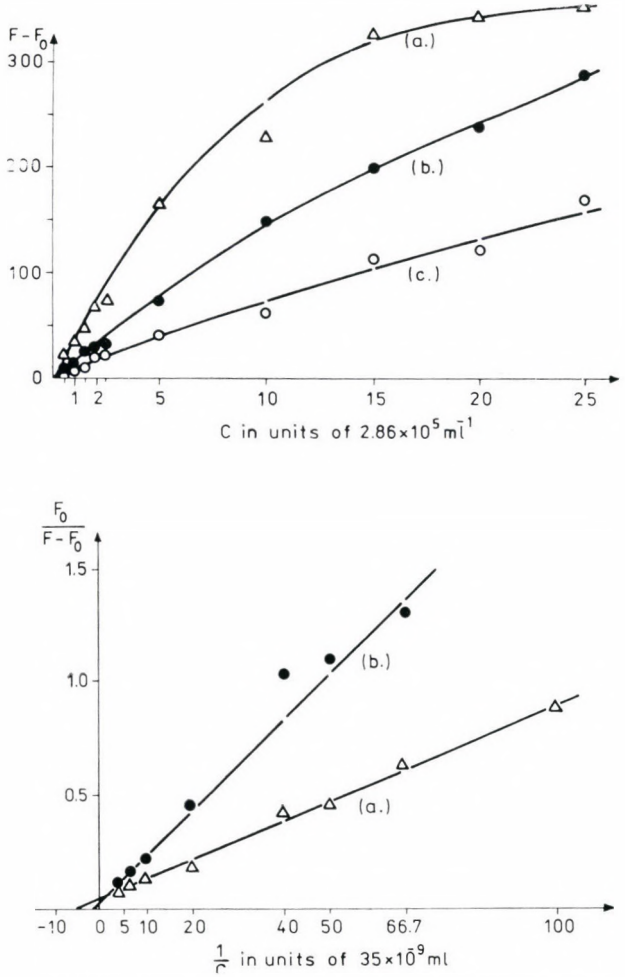

Fig. 1. Changes in fluorescence intensity as a function of cell concentration. Figures A and B show linear and double reciprocal plots, respectively. F is the fluorescence intensity at excitation and emission wavelengths of 520 and 600 nm, respectively, while F_0 is the value of F when there is no cell in the sample. Curves *a* and *b* represent leukemic cells labeled with 1.85×10^{-5} M EB. Fluorescamine concentration during the labeling was 0.5 mM for curve *a* and none for curve *b*. Curve *c* refers to normal cells labeled only with 1.85×10^{-5} M EB.

intercepts p and q were calculated. It was found that the values of p ranged from 0.05 to 0.11. Table 1 shows the values of intercept q (i.e. the value of $\alpha \sum_{i=1}^{n} K_i \zeta_i$) at different fluorescamine and EB concentrations, for normal and leukemic cells.

Energy transfer experiments: In order to calculate transfer efficiency it is required to determine two experimental parameters, F_A^0 and F_A^D, i.e., the fluorescence intensities (at 385 nm excitation and 600 nm emission wavelengths) of the sample when only acceptor, and when both donor and acceptor are present, as a function of cell concentration. By analogy with the study of EB binding kinetics, the concentration of EB does not change during the experiment while cell concenration is changed.

In calculations of F_A^0 and F_A^D one cannot compare two samples, with and without donor, because the presence of the donor, fluorescamine, affects the EB binding ability of cells (see curves a and b in Fig. 1A). That is why F_A^0 and F_A^D were calculated from the spectral properties of different samples labeled with fluorescamine, EB, and both of them, respectively. In experiments with double labeled cells the fluorescence intensisites were measured at 385/470, 385/600 and 520/600 nm excitation/emission wavelength giving the values of $(385/470)_A^D$, $(385/600)_A^D$ and $(520/600)_A^D$, respectively when both donor and acceptor were present. The values of $(385/470)_A^0$, $(520/600)_A^0$, $(385/470)_0^D$ and $(385/600)_0^D$ were available from spectral measurements of samples labeled with acceptor (EB) or with donor (fluorescamine).

Since the values of $(385/470)_A^D$ and $(520/600)_A^0$ are characteristic of the amount of fluorescamine and of EB, respectively, the values of F_A^D and F_A^0 were calculated in the following way:

$$F_A^D = (385/600)_A^D - (385/470)_A^D \frac{(385/600)_0^D}{(385/470)_0^D}$$

$$F_A^c = (520/600)_A^D \frac{(385/600)_A^0}{(520/600)_A^0}$$

The correction values, calculated from the spectra, were as follows:

$$\frac{(385/600)_0^D}{(385/470)_0^D} = 0.065$$

$$\frac{(385/600)_A^0}{(520/600)_A^0} = 0.060$$

Using the above calculation, the values for $(F_A^D/F_A^0 - 1)^{-1}$ can be plotted against the reciprocal values of cell concentration. This plot should result in straight lines (see Eq.(22)) as it is demonstrated in Fig. 2. According to Eqs (25) and (26), one of the two intercepts of the curve (t) is in reciprocal relationship with $\bar{E}$, i.e. the average transfer efficiency, while the other one (s) is proportional to q, as defined earlier.

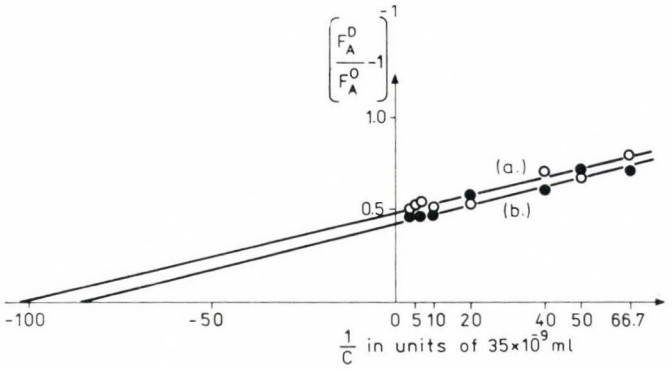

Fig. 2. Dependence of parameter $\left(\frac{F_A^D}{F_A^0}-1\right)^{-1}$ on the reciprocal value of cell concentration. Curves *a* and *b* refer to normal and leukemic cells, respectively. EB concentration was 1.85×10^{-5} M and the fluorescamine concentration, during labeling, was 0.25 mM in both cases.

Table 2

Variation of the intercepts $\frac{1}{t}$ *and* s *as a function of dye concentration in normal cells*

		Final ethidium bromide concentration in the samples					
		1.85×10^{-5} M		9.06×10^{-6} M		4.53×10^{-6} M	
		$\frac{1}{t}$	$s\times10^6$ [ml]	$\frac{1}{t}$	$s\times10^6$ [ml]	$\frac{1}{t}$	$s\times10^6$ [ml]
Fluorescamine concentration during the labeling	0.5 mM	2.62	1.75	4.0	3.7	4.95	5.8
	0.25 mM	2.08	3.6			2.41	7.4
	0.125 mM	2.20	0.53	2.62	1.3	2.58	3.9

Table 3

Variation of the intercepts $\frac{1}{t}$ *and* s *as a function of dye concentration in leukemic cells*

		Final ethidium bromide concentration in the samples					
		1.85×10^{-5} M		9.06×10^{-6} M		4.53×10^{-6} M	
		$\frac{1}{t}$	$s\times10^6$ [ml]	$\frac{1}{t}$	$s\times10^6$ [ml]	$\frac{1}{t}$	$s\times10^6$ [ml]
Fluorescamine concentration during the labeling	0.5 mM	2.41	4.9	3.65	4.2	3.39	4.7
	0.25 mM	2.32	3.25	2.63	3.4	2.87	4.3
	0.125 mM	1.72	3.7			2.44	3.1

In two sets of experiments, using normal or leukemic cells, the Förster-type energy transfer processes were investigated at different fluorescamine and EB concentrations.

The processed data of these experiments are shown in Tables 2 and 3. Table 2 shows the values of intercepts t and s at different fluorescamine and EB concentrations in the case of normal lymphocytes. Table 3 contains the values of the same intercepts for leukemic cells. The value of parameter s is given in units of ml which can easily be converted into M^{-1} as pointed out earlier in this paper.

Higher fluorescamine concentrations led to an increase in EB binding (values of s) with both normal and leukemic cells. A similar tendency is evident with respect to the values of $\frac{1}{t}$. Decrease of EB concentration increased the value of transfer efficiency.

Discussion

In the theoretical description of the kinetics of EB binding to cellular chromatin a model of multiple binding sites was applied. A linearized relationship among parameters characteristic of this model was established and the measurable fluorescence parameters were combined to facilitate the processing of experimental data in order to gain information about the cellular chromatin.

It is interesting that according to Table 1 both normal and leukemic cells undergo a significant change as a result of the fluorescamine treatment which leads to an increase in the value of parameter q, i.e. of EB binding ability of the cells.

According to the recent findings of Sondore et al. (1978) the EB binding characteristics for the purified chromatin seem to be the same as those for cells. However, there is some controversy in this respect, because not every cell is stained with EB. In our case it cannot be excluded that fluorescamine treatment increases the fraction of cells permeable to EB. An alteration in chromatin structure upon fluorescamine treatment may also occur. Fluorescamine binding can affect protein structure (Damjanovich et al., 1977) in such a way that the protein-nucleic acid interaction becomes weaker. This way result in a partial release of protein from DNA, i.e. in an increase in EB binding sites (α) on the chromatin, which would be detected as an increase in EB binding ability of cells, i.e. an increase of q.

As mentioned earlier, the values of intercept p ranged from 0.05 to 0.11 proving that the assumption that all the intercalated EB molecules have the same quantum yield is correct. The differences can be considered as a result of experimental errors.

Recently it has been shown that fluorescamine, covalently labeling the ε-amino groups of proteins and also other primary amines, and either EB or propidium iodide are suitable donor-acceptor pairs for studying RNA polymerase-DNA complexes or chromatin in vivo (Damjanovich et al., 1977; Steinkamp et al., 1974; Szöllősi et al., 1978).

A theoretical treatment of the kinetics of Förster-type energy transfer processes in the case of cells has shown that a) it is of practical use to define an average transfer efficiency, like the one given by Eq.(19) which contains structural information about the target of the applied fluorescent dyes, and that b) this average transfer efficiency can be calculated from data determined by experiments in which the cell concentration is varied, whereas the dye concentration is kept constant. A double reciprocal plot for the evaluation of parameters containing $\bar{E}$ as well is given by Eq.(22) which stands for the well-known form of Eq.(20). Eq.(22) makes it possible to determine not only the parameter t which contains information about cell structure, but also the parameter s which is proportional to q, i.e. the binding ability of EB molecules to the cells. Eq.(26) shows that the value of s, because of the value of η for EB, is about one order of magnitude higher than that of q.

An experimental verification of this theoretical consideration is given in Fig. 2 showing that experimental data processed according to Eq.(22) yield straight lines indeed. The values of the intercepts t and s for different dye concentrations are given in Tables 2 and 3 for normal and leukemic cells, respectively.

It is of interest to note that normal and leukemic cells react differently to changes in EB concentration. The elevation of EB concentration has practically no effect on s in the case of leukemic cells, while with normal cells it results in a marked decrease of s, i.e. the EB binding ability of cells (there is no change in η, defined by Eq.(8), which is also involved in the parameter s). This difference between the two kinds of cells can be interpreted as follows: The EB binding of leukemic cells is in good agreement with the theoretical treatments, which predict no change in s, upon the change of EB concentration. This prediction is valid as long as Eqs (3) and (4) hold, i.e. as long as there is no saturation for any class (i) of EB binding sites. However, when a subpool (the one having the highest K_i) is almost saturated, Eqs (3) and (4) do not hold any longer for this pool and the following kind of EB concentration dependence of q appears: When the concentration of EB is increased, the pools having the highest K_i-s become more saturated, and only the remaining EB binding sites with the next highest K_i can be monitored by the kinetics. This way one can get a decreasing value of q on increasing EB concentration. This is the case with the normal cells.

The last columns in Tables 2 and 3 show that the normal cells have a higher EB binding ability than the leukemic cells at the lowest EB concentration applied. These facts, along with the above discussed characteristics of the parameter q, indicate that *a)* the number of EB binding sites (α) is lower for normal than for leukemic cells (that is why there is no observable subpool saturation for leukemic cells), and that *b)* the affinity of EB binding sites is higher with the normal cells than with the leukemic ones (see the last columns of Tables 2 and 3).

As to the variation of parameter t with the concentrations of fluorescamine and EB, two remarks can be made: *a)* The value of 1/t does not show the linear relationship with the fluorescamine concentration as Eq.(25) would predict it. Accepting that C_f is proportional with the fluorescamine concentration applied, there is one explanation that would account for this nonlinearity: The higher the

fluorescamine concentration, the higher the portion of chromatin protein released, i.e. the higher the portion of bound fluorescamine that does not take part in the energy transfer processes. *b)* An increase in the concentration of EB leads to a decrease of 1/t indicating that the average transfer efficiency, $\bar{E}$, also decreases. This can be the result of EB binding to double stranded nucleic acids having no significant amount of bound proteins. This phenomena, elevating the amount of bound EB molecules that do not take part in the Förster-type energy transfer processes, result in a decrease of the average transfer efficiency, $\bar{E}$. Another possible explanation to account for this effect is that the higher the EB concentration, the higher the fraction of proteins released from the chromatin by the intercalated EB.

The above theoretical and experimental investigations show that a careful test of binding kinetics and of the interaction of dyes is necessary when fluorescent dyes are applied to cells in order to find structural differences. From a comparison of the data in Tables 2 and 3 it can be seen that some circumstances are appropriate to find differences in the two kinds of cells, lymphocytes and lymphoblasts, while others are not.

It should be noted that the above kinetic treatment can also be adapted to flow fluorimetry to extend the method to get not only the mean values but also their distribution over single cells.

Thanks are due to Mrs Ágnes Gara for the excellent technical assistance and to Mr Árpád Szekeres for drawing the figures.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 21—28 (1980)

A Kinetic Study of the K^+ Activated ATPase of Myosin Subfragment-1

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(Received June 20, 1979)

The K^+ activated ATPase activity of myosin subfragment-1 was measured under different conditions and enzyme kinetic parameters were calculated. The logarithm of K_m varies linearly with ionic strength down to very low KCl concentrations, the logarithm of k_2 vs. ionic strength, on the other hand, considerably deviates from linearity at low concentrations of KCl.

ADP is a competitive inhibitor, like myosin ATPase, and with practically the same inhibitor constant.

The energetical parameters of the decomposition (to products and enzyme) of the S-1—ATP complex are partically the same as those for myosin, the parameters of its formation, however, differ from the corresponding values for myosin:

$\Delta S_1^{\ddagger}$ is considerably, $\Delta H_1^{\ddagger}$ significantly higher in the case of myosin. This may be the result of some kind of interaction of the two heads of myosin.

Introduction

In the literature there are contradictory assumptions as to the significance of the "two headedness" of myosin. One of them is referred to as the Bagshaw—Trentham mechanism, which is based on the transient kinetic studies of myosin ATPase activated by Mg^{2+} ions, and assumes only one pathway of ATP hydrolysis (Bagshaw and Trentham, 1973; 1974; Mannherz at al., 1974). The other one, suggested by Tonomura and Inoue (1974), Arata et al. (1975) assumes two different routes for the two heads of myosin — but no agreement has been reached as yet on whether the hydrolysis step is the same for both heads.

In this paper, which is a continuation of our similar report on myosin (Kelemen, Magyar, 1975; Kelemen, 1979), we deal with the kinetics of K^+ ion activated ("EDTA activated") ATPase of myosin subfragment-1.

The papain cleveage of myosin yields globuar subfragment-1, which is a homogeneous and monodisperse molecule according to gelfiltration and ultracentrifugation (Stone, Perry, 1973), its specific activity (on molecular basis) being twice as high as that of myosin (Nauss at al., 1969).

Abbreviations: S-1, papainic fragment of myosin.

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It seems of interest to know how S-1 behaves in a kinetically simple system as compared to the two-headed myosin, and how the reaction mechanism of S-1 changes under circumstances when these reaction parameters of myosin cannot be measured.

Materials and methods

Myosin was prepared as described previously (Kelemen, Mühlrad, 1971). Subfragment-1 was isolated from myosin by papain digestion. About 20 mg protein/ml in 20 mM sodium phosphate (pH 6.5), 3 mM EDTA, 20 mM KCl and 5 mM cysteine was treated with 0.1 mg/ml papin for 10 min at 25 °C. Digestion was terminated by addition of iodoacetic acid (Bálint et al., 1975). After dialysis the digest was fractionated on DEAE-cellulose to give purified subfragment-1.

The purified preparations were analyzed for homogeneity by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

The myosin concentration was determined by the biuret method of Gornall et al. (1949) and the subfragment-1 concentration was estimated using a value of $E^{1\%}_{280\,nm} = 8.16$.

The molecular weights of myosin (Tonomura et al., 1966) and subfragment-1 (Lowey, Steiner, 1972) were assumed to be 5×10^5 and 1.2×10^5 daltons, respectively.

ATPase activities were measured as described previously (Kelemen, Magyar, 1975). Conditions for K^+ dependent ATPase assays were: 40 mM Tris-HCl (pH 8), 9 mM EDTA, 6–10 ug/ml protein, 2–52 mM KCl. In each series of experiments the ATP concentration varied around the actual K_m value. The measurments were carried out generally at 20 °C (or 0, 10, 30 °C) using 8 ml samples. Incubation was terminated by addition of sulfuric acid. After mixing with charcoal suspension, the reaction mixtures were filtered and the radioactivity of ^{32}P in the filtrate was measured in a Beckman LS-100 C liquid scintillation counter.

The results were analyzed by conventional Michaelis – Menten kinetics.

Statistical analysis was made by a computer program which fitted a linear regression curve on the experimental data, computed the correlation coefficient and gave, together with the evaluation the standard deviation of V and K_m, which is less than 10 per cent.

Results

The K^+ activated myosin ATPase follows a simple Michaelis kinetics (Lymn and Taylor, 1970; Kelemen and Mühlrad, 1971; Taylor, 1977). As shown by Mandelkow and Mandelkow (1973), the value of k_{-1} (the rate constant of dissociation) is negligible. This allows the calculation of the rate constant of [ES] complex formation (k_1) from V (i.e. k_2) and K_m:

$$k_1 = \frac{k_2 + k_{-1}}{K_m} \approx \frac{k_2}{K_m} \tag{1}$$

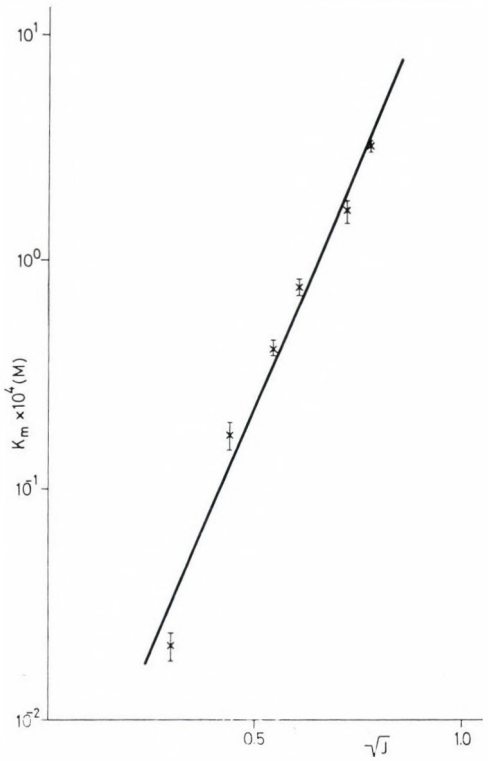

Fig. 1. Depedence of log K_m on the square root of KCl concentration. Conditions of ATPase activity measurements see methods and text. (The statistical analysis was made by a computer program which fitted a linear regression curve. The parameters of the fitted line are: a = −6.84 and b = 4.39, n = 270.)

In our paper on myosin (Kelemen, Mühlrad, 1971; Kelemen, Magyar, 1975) we found that the values of log K_m, log k_2, and log k_1 (as calculated from equation 1) depended linearly on ionic strenght in the range of 0.3 − 0.7.

As shown in Figs 1, 2 and 3, if we extend the measurements with S-1 to lower ionic strength, the linearity of log K_m still prevails, but log k_2 strongly deviates from linearity, and so does log k_1. (The value of k_{-1} is assumed to be negligible.) In view of this behaviour we evaluated the energetical parameters of the formation and decomposition steps only at 0.3 ionic strength. Using that ionic strength, k_1 can be unambiguosly calculated, and data for comparison from our former work (Kelemen, Magyar, 1975) can be applied.

Figs 4 and 5 show the temperature dependence of the rate constants of [ES] complex formation and decomposition. As seen in Fig. 4, the Arrhenius plot of k_1 constants deviates at 0 °C. For this reason the curve used for the calculation of

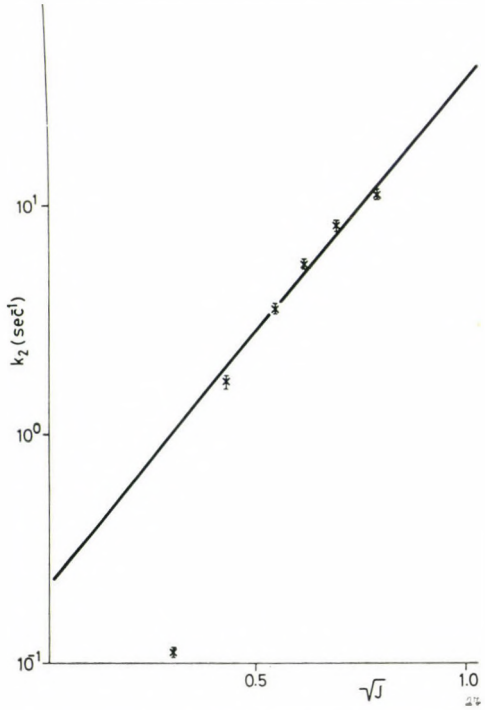

Fig. 2. Dependence of log k_2 on the square root of KCl concentration. For details of the measurements and evaluation see text and Fig. 1. (The regression curve was fitted to the values obtained at 0.3; 0.4; 0.5 M KCl concentration. The parameters of the fitted line are: a = −0.623 and b = 2. 149, n = 135.)

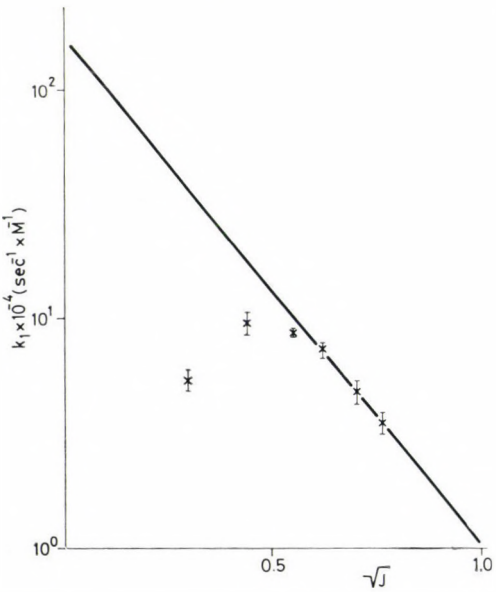

Fig. 3. Dependence of log k_1 on the square root of KCl concentration. For evaluation of k_1 see text and Fig. 1. (The parameters of the fitted line are: a = 6.20 and b = −2.17, n = 135.)

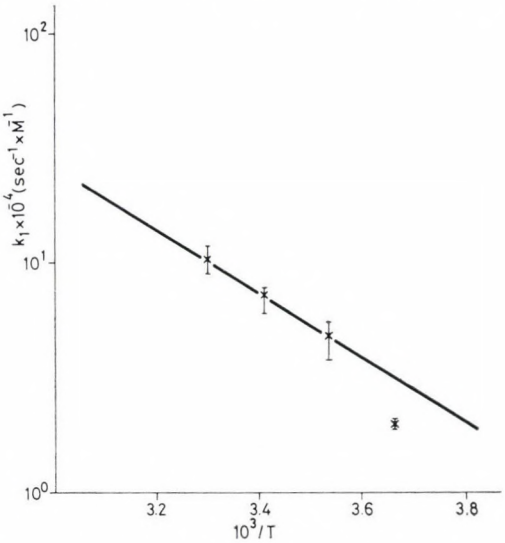

Fig. 4. Dependence of log k_1 on the reciprocal of absolute temperature. For evaluation of k_1 see text. The regression curve was fitted to the values obtained at 10, 20 and 30 °C. (The parameters of the fitted line are: $a = 9.64$ and $b = -1.40$, $n = 135$.)

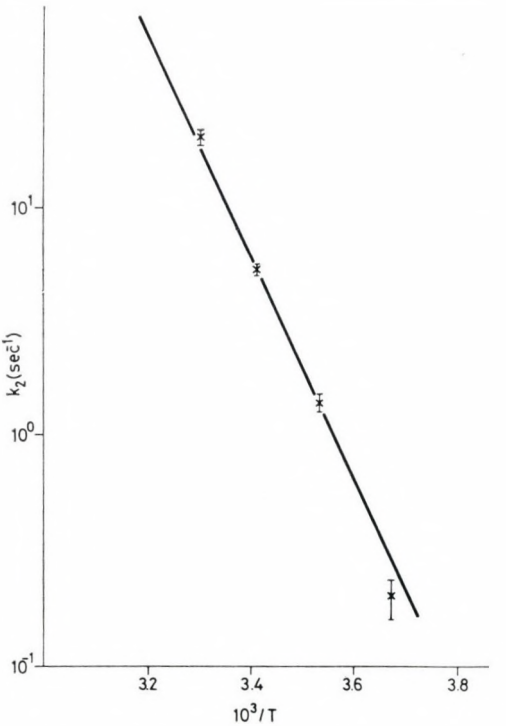

Fig. 5. Dependence of log k_2 on the reciprocal of absolute temperature. For details of the measurements and evaluation see text and Fig. 4. (The parameters of the fitted line are: $a = 17.7$ and $b = -4.97$, $n = 135$.)

energetical parameters was fitted only to the values obtained at 10, 20 and 30 °C, respectively; for k_2 (Fig. 5) the curve was fitted similarly.

In Tables 1 and 2 the energetical parameters of myosin and S-1 as well as their differences are shown (calculations described in Kelemen, Magyar, 1975). The activation entropy ($\Delta S_1^{\ddagger}$) and activation energy ($\Delta H_1^{\ddagger}$) of enzyme – substrate

Table 1

Energetical parameters derived from the temperature-dependence of k_1 for myosin (Kelemen, Magyar, 1975) *and for S – 1* (this paper)

	Myosin	S – 1
$\Delta H_1^{\ddagger}$ kcal · mol^{-1}	11.6	6.4
$\Delta H_1^{\ddagger}$ myosin – $\Delta H_{1\,S-1}^{\ddagger}$	5.2	
$\Delta S_1^{\ddagger}$ cal · mol^{-1} . degree^{-1}	4.2	−14.3
$\Delta S_1^{\ddagger}$ myosin – $\Delta S_{1\,S-1}^{\ddagger}$	18.5	
$\Delta S^{\ddagger}$ at T = 293 K		

complex formation are much higher (by 18.5 cal . mol^{-1} · degree^{-1} and 5.2 kcal · · mol^{-1}, respectively) for myosin than for S-1 (Table 1).

On the other hand, there is hardly any difference between the activation energies and entropies of the decomposition of S-1-ATP complex and the myosin-ATP complex (Table 2).

As far as the effect of ADP on S-1 ATPase (Fig. 6) is concerned, we have found that, like in the case of myosin (Kelemen, 1979), it is a competitive inhibitor.

The inhibitor constant (K_I) is 1.14×10^{-4} M (in 0.3 M KCl at pH 8).

Table 2

Energetical parameters derived from the temperature-dependence of k_1 for myosin (Kelemen, Magyar, 1975) *and for S – 1* (This paper)$_2$

	Myosin	S – 1
$\Delta H_2^{\ddagger}$ kcal · mol^{-1}	24.0	22.7
$\Delta H_2^{\ddagger}$ myosin – $\Delta H_{2\,S-1}^{\ddagger}$	1.3	
$\Delta S_2^{\ddagger}$ cal · mol^{-1} . degree^{-1}	27.5	22.5
$\Delta S_2^{\ddagger}$ myosin – $\Delta S_{2\,S-1}^{\ddagger}$	5.0	
$\Delta S_2^{\ddagger}$ at T = 293 K		

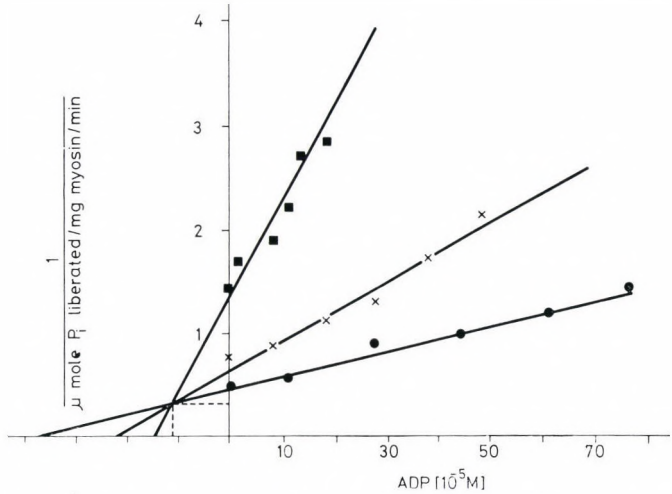

Fig. 6. Dependence of the reciprocal of the specific activity of K^+ ATPase of S-1 on ADP concentration at different ATP concentrations. For details of ATPase measurements see Methods. ATP concentrations: ■, 1.37×10^{-5}, ×, 4.25×10^{-5}; ●, 7.4×10^{-5} M

Discussion

The data presented in this paper show that from 0.3 M K^+ concentration upwards, the values of k_1 (rate constant of formation) k_2 (rate constant of decomposition) and K_m for the S-1-ATP complex change in the same way as in the case of myosin (Kelemen, Magyar, 1975). This may be due to primary salt effect.

At lower ionic strength, while log K_m continues to change linearly with ionic strength, log k_2 clearely deviates from linearity. If the linearity of log K_m with nonlinear change of log k_2 is considered to be due to a nonnegligible value of k_{-1}, this value of k_{-1} can be evaluated for the low ionic strength experiments. Such a calculation gives values of 0.44 sec^{-1} and 0.91 sec^{-1} for 0.12 and 0.02 M KCl, respectively, which are in good agreement with the values found by Mandelkow and Mandelkow (1973) by direct measurement based on a fluorescence method at similar ionic strength of NaCl. In any case, the nonlinear behaviour of log k_2 for S-1 (and possibly of log k_1) shows that the mechanism of the reaction changes at lower ionic strength.

ADP is a competitive inhibitor, just as in the case of myosin. The inhibitor constants in 0.3 M KCl (at pH 8) of myosin and S-1 are practicaly the same, for myosin $K_I = 1.65 \times 10^{-4}$, for S--1 $K_I = 1.14 \times 10^{-4}$ M. This suggests that, in the presence of ADP, the mechanism of competition is similar in both cases.

As to the mechanism of action of the K^+ activated ATPase of myosin and S-1, it should be pointed out that the molecular activity of the two-headed myosin is practically double that of the oneheaded S-1 (Nauss et al., 1969), thus it is the

same perhead. The energetical parameters of the decomposition of the enzyme-substrate complex to product and enzyme are practically the same, as shown in this paper. Thus the molecular mechanism of the catalytic step seems to be the same.

On the other hand, there are conspicuous differences in energetical parameters of enzyme-substrate complex formation: for myosin $\Delta S_1^{\ddagger}$ is considerably higher (by round 20 cal . mol^{-1} . $degree^{-1}$) and $\Delta H_1^{\ddagger}$ is also higher as for S-1 (by 5 kcal . mol^{-1}). In the case of myosin, the increase of $\Delta H_1^{\ddagger}$ is amply overcompensated by the strong increase in entropy of formation. Thus the rate of formation of enzyme-substrate complex is considerably higher for myosin. This may possibly be due to a cooperative interaction between the two heads.

We are indebted to Prof. N. A. Biró for valuable discussion and correction of the manuscript.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 29—37 (1980)

Investigation of the Active Center and Catalytic Mechanism of Porcine Kidney Aminoacylase: a Model of the Active Center

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(Received May 25, 1979)

Porcine kidney aminoacylase (E.C. 3.5.1.14) is inhibited neither by phenylmethylsulfonylfluoride nor by alkylating agents (iodoacetate or iodoacetamide). Therefore reaction mechanisms including the formation of acylenzyme through seryl or cysteinyl side chains are ruled out.

The enzyme is a metalloprotein that can be inactivated by EDTA and in which Co^{2+} is an equivalent substitute for the Zn^{2+} ion.

The two SH groups/subunit of aminoacylase exhibit different reactivities to p-hydroxymercuribenzoate. Modification of the less reactive SH group reversibly inactivates the enzyme. We suggest that this cysteinyl side chain is situated in the active center or in its immediate vicinity.

On the basis of our results we suppose a close similarity between aminoacylase and carboxypeptidase A with respect to their active center and catalytic mechanism.

Introduction

According to the studies of Kördel and Schneider (1977a, b), each subunit of porcine kidney aminoacylase (E.C. 3.5.1.14) contains one Zn^{2+} ion which is bound via histidyl side chains. According to these authors, under certain conditions, the blocking by N-ethylmaleimide of the more reactive SH group of the two SH groups/subunit will lead to the inactivation of the enzyme. On the basis of the protective effect of competitive inhibitors (N-tosyl-L-alanine, DL-norleucine) it may be supposed that this cysteinyl side chain is situated near the highly hydrophobic substrate binding site of aminoacylase (Kördel, Schneider, 1976a).

The enzyme can be inactivated also by 1H-diazotetrazol or bromosuccinimide. In this case one tryptophan residue reacts in each subunit and neither the substrate nor the inhibitors provide protection (Kördel, Schneider, 1976b). According to Löffler et al. (1975) the enzyme can be inactivated also by TPCK. From the pH dependence of the inactivation they inferred the presence of an amino group that may participate in the binding of the carboxylate-anion of the

Abbreviations: EDTA, ethylenediaminetetraacetic acid, disodium salt; IAA, iodoacetate; IAAm, iodoacetamide; PMB, p-hydroxymercuribenzoate; PMSF, phenylmethylsulfonylfluoride; TPCK, L-(1-tosylamido-2-phenyl)ethylchlormethylketone.

substrate. The hydrophobic region of the active center also participates in substrate binding (Moravcsik et al., 1971a, b; Kördel, Schneider, 1975).

The mechanism of the catalytic reaction has been studied by Ötvös et al. (1971). They suggested that the hydrolysis of the acylaminoacid proceeds via an acylenzyme intermediate and in the rate-limiting step of the reaction the carbonyl-C atom of the acylamino group is subjected to nucleophilic attack.

In this work we studied whether the active center of porcine kidney aminoacylase contains any activated OH or SH groups, and whether the Zn^{2+} ion can be replaced by Co^{2+}. On the basis of our results and the data in the literature we constructed a hypothetical model of the active center of porcine kidney aminoacylase, and from this we suppose a close similarity between aminoacylase and carboxypeptidase A with respect to the structure of the active center and the catalytic mechanism.

Materials and methods

Aminoacylase, isolated from porcine kidney, was a salt-free lyophilized preparation (Reanal Factory of Laboratory Chemicals, Budapest, Hungary). Its specific activity, measured at 25 °C, pH 7.0, with N-acetyl-L-methionine as substrate was 2500 ± 200 units/mg protein. For the calculations we used a molecular weight of 86 000 (Kördel, Schneider, 1976a).

N-acetyl-L-alanine and N-acetyl-L-methionine were commercial preparations of p.a. quality (Reanal).

Ethylenediaminetetraacetic acid (disodium salt), phenylmethylsulfonylfluoride, p-hydroxymercuribenzoate, iodoacetic acid and iodoacetamide were Serva Feinbiochemica (Heidelberg, GFR) products.

All other chemicals used were of analytical grade (Reanal).

Protein determination was performed according to Lowry et al. (1951) as modified by Schacterle and Pollack (1973).

Measurement of aminoacylase activity was based on the determination of L-methionine liberated in the reaction (Birnbaum et al., 1952). The colorimetric ninhydrin method was used for the measurements (Moore, Stein, 1948). The reaction mixture contained aminoacylase, 0.002 mg/ml; N-acetyl-L-methionine, 0.015 M; potassium phosphate, 0.1 M, pH 7.0. For inhibition studies, incubation times were chosen according to the expected activity. The reaction was terminated by heat treatment for 3 minutes in a hot water bath. The controls containing only substrate plus buffer or enzyme plus buffer were treated in an identical manner. Activity was determined from aliquots (0.1 ml) of the samples (Spies, 1957). L-methionine concentration was determined from absorbance values at 570 nm with the help of a calibration curve. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmole of L-methionine in 1 hour, at 25 °C, pH 7.0.

Reactivating effect of Zn^{2+} and Co^{2+} ions: Enzyme inhibited by EDTA was dialyzed against 0.05 M Na-veronal buffer for 48 hours at +4 °C, then incubated

for 15 minutes at 37 °C in the presence of $10^{-4} - 2 \times 10^{-3}$ M Zn^{2+} or Co^{2+} ions. For the determination of the K_M value, the reactivated samples were dialyzed against water and lyophilized. Native aminoacylase treated in the same way was used as control.

The effect of PMSF: PMSF was dissolved in acetonitryl. Throughout the experiments 0.1 M potassium phosphate buffer was used. Protein concentration in the individual series of experiments was 1.17×10^{-6} or 1.17×10^{-5} M, PMSF concentration was $10^{-5} - 10^{-3}$ M. After incubation for 10 minutes the residual activity of the samples was determined.

The effect of alkylating agents: Iodoacetic acid or iodoacetamide was used as an alkylating agent. Incubation was performed at 37 °C in 0.1 M potassium phosphate buffer, pH 7.0 or 8.5. In the different experiments protein concentration was $4-12 \times 10^{-5}$ M, iodoacetic acid concentration $9-40 \times 10^{-4}$ M and iodoacetamide concentration 9×10^{-4} M, the molar ratio of the reagent to the enzyme was at least 20 : 1. The reaction was stopped by diluting the reaction mixture 100-fold. Residual activity was determined from the diluted mixture.

Carboxymethylation was studied in a pH-stat at pH 7.0 and 25 °C. Protein concentration was 5×10^{-5} M, iodoacetate concentration $2.5-4 \times 10^{-3}$ M, the molar ratio of the reagent to the enzyme was at least 50 : 1. Titrations with 0.001 N NaOH were carried out in a TTT 1 type automatic titrator (Radiometer, Copenhagen) equipped with an ABU 12 autoburette.

The effect of PMB: The number of SH groups was determined in the presence of 6.7 M urea at 25 °C, pH 7.0, in 0.1 M potassium phosphate buffer, by the spectrophotometric method of Boyer (1954). An extinction coefficient of 7.6×10^3 M^{-1} cm^{-1} at 250 nm was used for the calculations.

For the native enzyme, the time course of mercaptide formation was determined by difference spectrophotometry.

For studying the reversibility of inhibition, aminoacylase containing two mercaptide groups per subunit was prepared. The inactive enzyme was incubated with 2-mercaptoethanol added in a 1.5-fold excess (regarding the number of mercaptide groups) for 15 minutes at 25 °C in 0.1 M potassium phosphate buffer, pH 7.0. The reaction mixture was subjected to gel filtration on a Sephadex G-25 column.

Photometric measurements were carried out in a Pye Unicam SP 1800 spectrophotometer (Pye Unicam Ltd., Cambridge, England) to which an AR 25 type recorder was connected.

Results

The role of metal ions in aminoacylase activity

Chelating reagents, such as EDTA, are known to inactivate porcine kidney aminoacylase at a well measurable rate. In our experiments $3-6 \times 10^{-5}$ M protein and $10^{-3} - 10^{-2}$ M EDTA were used. A typical series of experiments is shown in

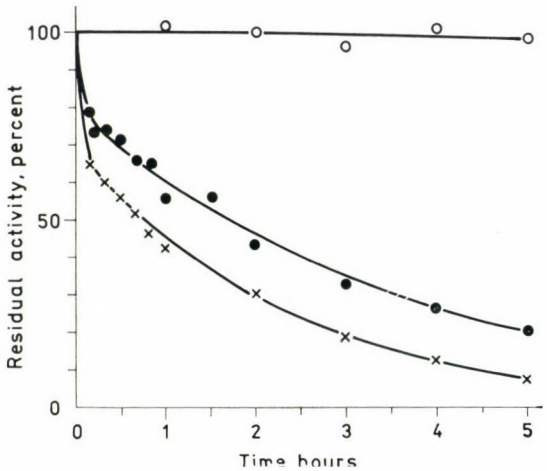

Fig. 1. Inactivation of aminoacylase by EDTA at 37 °C in 0.1 M K-phosphate buffer, pH 7.0. Protein concentration 6×10^{-5} M. ○, control; ●, 10^{-3} M EDTA; ×, 10^{-2} M EDTA

Fig. 1. Inhibition brought about by EDTA is not reversed by extensive dialysis (48 hours). Zn^{2+} or Co^{2+} ions practically fully reactivated the inhibited enzyme (Table 1). This means that reactivation is a relatively fast process as compared to inactivation.

After the removal by means of dialysis of the excess metal ions, the K_M values of the samples reactivated either by Zn^{2+} or by Co^{2+} were determined with N-acetyl-L-methionine as substrate. The K_M values obtained were practically identical with that of the control ($K_M = 3.6 \pm 0.2 \times 10^{-3}$ M).

Table 1

Effect of Zn^{2+} and Co^{2+} ions on aminoacylase inactivated by EDTA

0.3×10^{-4} M enzyme inactivated by EDTA (residual activity 3.5%) was dialyzed against 0.05M Na-veronal buffer (pH 7.0) for 48 hours at +4 °C, then treated with Zn^{2+} or Co^{2+} for 15 min at 37 °C in the same buffer (pH 7.0). Activity was measured with N-acetyl-L-methionine as substrate. 100% activity = 2450 units/mg protein

Metal ion added ($M \times 10^4$)		Activity recovered (%)	
Zn^{2+}	Co^{2+}	"Zn-acylase"	"Co-acylase"
0.94	1.0	68.4	98.0
1.85	2.0	100.7	101.5
3.7	3.0	104.0	84.0
7.52	6.0	85.9	108.6
18.49	15.0	96.3	110.3

Studies of the SH and OH groups in aminoacylase

a) *Effect of PMB*

Both in native enzyme and in that treated with 6.7 M urea, approximately 4 SH groups/dimer were titrated. In the native enzyme, about 2 SH groups/mole reacted with the reagent already in the first 15 seconds of the reaction. The rest of the SH groups reacted considerably slower (Fig. 2/A).

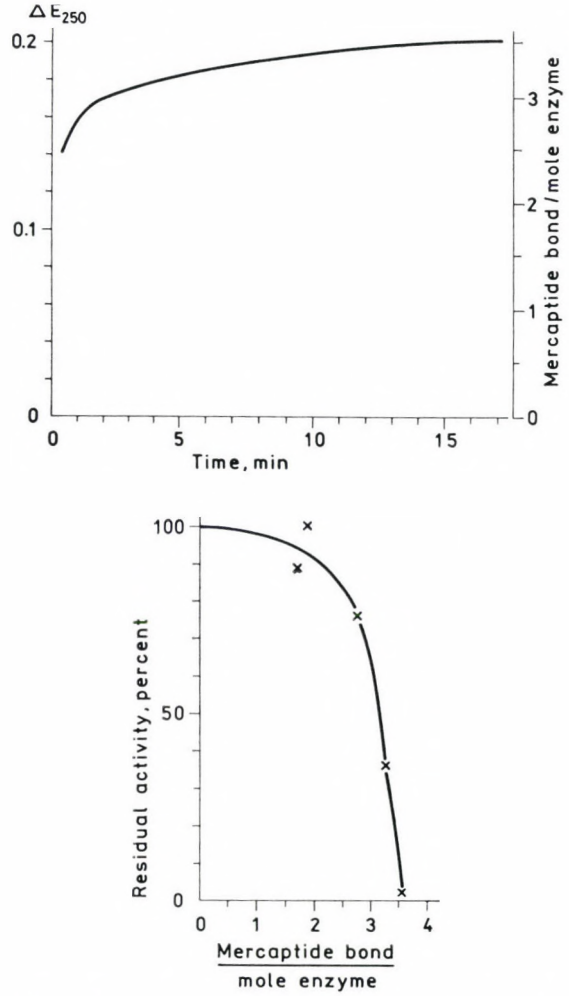

Fig. 2. Effect of PMB on aminoacylase at 25 °C in 0.1 M K-phosphate buffer, pH 7.0. *A:* Reaction of aminoacylase with PMB. Protein concentration 3.9×10^{-6} M, PMB concentration 1.56×10^{-5} M. *B:* Change in aminoacylase activity as a function of the number of mercaptide groups bound

Table 2

Comparison of the V_{max} and K_M values of native aminoacylase and aminoacylase inhibited by PMB, then reactivated by 2-mercaptoethanol

The inactive enzyme containing 4 mercaptide groups/mole was treated with 2-mercaptoethanol (for details see Methods)

Substrate	Native enzyme		Reactivated enzyme	
	V_{max} (unit/mg)	K_M ($M \times 10^3$)	V_{max} (unit/mg)	K_M ($M \times 10^3$)
N-acetyl-L-alanine	425	15.0	437	17.0
N-acetyl-L-methionine	2890	3.0	2825	2.2

Table 3

Effect of phenylmethylsulfonylfluoride and alkylating agents on the activity of porcine kidney aminoacylase

Enzyme concentration ($M \times 10^5$)	PMSF ($M \times 10^5$)	IAA ($M \times 10^5$)	IAAm ($M \times 10^5$)	Residual activity (%)
0.117	1	—	—	[1+]84.2
	10	—	—	90.3
	100	—	—	91.6
1.17	10	—	—	102.7
	100	—	—	93.1
10.6	—	91.1	—	[2+]107.3
	—	229	—	107.9
	—	228	—	[3+]101.8
10.95	—	—	90	[2+]98.4

[1+] 0.1 M K-phosphate buffer, pH 7.5; 30 °C; incubation for 10 min.
[2+] 0.1 M K-phosphate buffer, pH 7.0; 37 °C; incubation for 2 hours.
[3+] 0.1 M K-phosphate buffer, pH 8.5; 37 °C; incubation for 2 hours.

The enzyme was inactivated by PMB. When enzyme activity was plotted against the number of mercaptide groups bound (Fig. 2/B), the individual cysteinyl side chains were found to be functionally different. The blocking of 2 SH groups/mole did not influence activity; for inactivation, the blocking of two more SH groups/mole was needed. As the enzyme is a dimer (Kördel, Schneider, 1976a), it seems that the blocking of 1 cysteinyl side-chain – that of lower reactivity – per subunit inactivates the enzyme. After treatment with 2-mercaptoethanol, the modified enzyme regained practically all of its original catalytic activity. The V_{max} and K_M values of the reactivated enzyme for the substrates N-acetyl-L-alanine and N-acetyl-L-methionine were practically identical with the corresponding values of the untreated enzyme (Table 2).

b) *Effect of PMSF and alkylating agents*

Under the reaction conditions applied, incubation with these reagents did not alter the activity of the enzyme as compared to that of the control (Table 3). During alkylation in a pH stat, no carboxymethylation could be demonstrated.

Discussion

The structure of porcine kidney aminoacylase in general, that of its active center in particular and the mechanism of the catalytic reaction have only lately become the subject of more detailed studies. Ötvös et al. (1971) have studied the mechanism of the reaction catalyzed by aminoacylase. From the reaction mechanism of common occurrence among esterolytic enzymes, they supposed that the reaction proceeds via an acylenzyme intermediate. We have studied whether an activated OH or SH group of the enzyme participates in the formation of the suggested acylenzyme intermediate. We found that the enzyme was not inactivated either by PMSF, or by iodoacetate or iodoacetamide. From these results we draw the conclusion that the active center of aminoacylase does not contain any activated SH or OH groups, and this fact in all probability eliminates the possibility of any direct analogy between the mechanism of action of aminoacylase and that of the main endopeptidases (trypsin, chymotrypsin, subtilisin, papain, ficin, etc.). For the sake of comparison we mention that the rate constant for the inactivation of chymotrypsin by PMSF is $k = 14{,}900\ M^{-1}min^{-1}$ at 25 °C, pH 7.0 (Fahrney, Gold, 1963) and the rate constant for the alkylation of papain by IAAm is $k = 976\ M^{-1}s^{-1}$ and by IAA, $k = 20\ M^{-1}s^{-1}$ at 25 °C, pH 10.35 (Halász, Polgár, 1976, 1977).

The enzyme is inhibited by EDTA and the inhibited enzyme can be reactivated not only by Zn^{2+} but also by Co^{2+} ions. In both cases, the K_M value of the reactivated enzyme measured with N-acetyl-L-methionine as substrate is identical with that of the control sample. These results support the result of Kördel and Schneider (1977b), that the enzyme is a metalloprotein containing a closely bound metal ion that seems to be essential for the catalytic activity. In addition, our results demonstrate that Co^{2+} is an equivalent substitute for Zn^{2+}.

It is interesting to note that of the 2 SH groups/subunit, the one that is seemingly not involved in the catalytic activity reacts faster with PMB than does the one which is supposed to play a role in the catalytic process, while with Ellman's reagent, N-ethylmaleimide or carboxypyridinedisulfide as reagents it is just the other way round (Kördel, Schneider, 1976a). On the contrary, we could not demonstrate inactivation or carboxymethylation by alkylating agents even under conditions in which the reaction of activated SH groups is very fast (Boross, Cseke, 1967; Halász, Polgár, 1976, 1977). This fact, together with the observation that the enzyme is not inhibited by PMSF, practically precludes the assumption that catalysis proceeds via an acylenzyme intermediate.

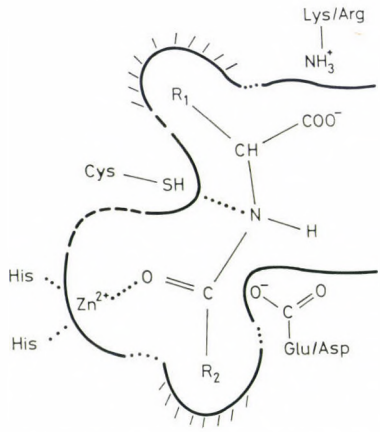

Fig. 3. Hypothetical model of the active center of porcine kidney aminoacylase

The absence of active OH or SH groups and the presence of an essential, bivalent cation in aminoacylase raise the possibility of a similarity between aminoacylase and carboxypeptidase A, with respect to their active center and catalytic mechanism. This is also supported by the fact that, like in carboxypeptidase A (Coleman, Vallee, 1961), Co^{2+} is an equivalent substitute for Zn^{2+} in aminoacylase. If we accept the existence of such a similarity, then, by analogy to dipeptide hydrolysis catalyzed by carboxypeptidase A (Lipscomb et al., 1968, 1970; Hartsuck, Lipscomb, 1971; Quiocho, Lipscomb, 1971; Reeke et al., 1967; Quiocho et al., 1971), the possible role of the metal ion may be to increase the susceptibility to nucleophilic attack of the carbonyl group of the substrate by polarization. The alleged nucleophilic reaction partner may be a carboxylate anion of an amino acid side chain of the enzyme; for this, however, we do not have experimental evidence yet.

We suggest that the role of the cysteinyl side chain which is seemingly essential for catalytic activity might be identical with that of the tyrosyl-248 side chain of carboxypeptidase A, i.e. it may act as a proton donor in the reaction by way of its proton being bound to the amino nitrogen when the amide bond is split. A hypothetical model of the active center of aminoacylase is presented in Fig. 3.

The authors express their gratitude to Dr Gertrud Szabolcsi for her valuable suggestions and help in the preparation of the manuscript, and to Mrs Erzsébet Dala and Mrs Margit Zumpf for their outstanding technical assistance.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 39—47 (1980)

Isolation of γ-L-Glutamyl-Taurine from the Protein Free Aqueous Extract of Bovine Parathyroid Powder*

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(Received May 11, 1979)

The so far unknown γ-L-glutamyl-taurine has been isolated by submitting the protein free aqueous extract of bovine parathyroid powder to gel filtration, ion exchange chromatography, paper chromatography, paper electrophoresis at pH 2, and at pH 6.5. The structure has been derived from the composition and electrophoretic mobility of the dipeptide, taking into account that glutamic acid liberated on acid hydrolysis could be degraded by L-glutamic acid decarboxylase.

Introduction

The protein free aqueous extract of bovine parathyroid powder was shown by Feuer et al. (1977) to have an influence on serum vitamin A level in rats. Furthermore, oral administration of this extract to rats after irradiation by 850 R or 1000 R resulted in significant increase of survival (Feuer, Ormai, 1978). In this paper the isolation of a new compound, responsible for the effects mentioned above, is described.

Materials and methods

The protein free aqueous extract of bovine parathyroid powder was prepared as described by Feuer et al. (1977).

L-Glutamic acid decarboxylase (EC 4.1.1.15) was purchased from Worthington (GLD 46CO48). The other materials and reagents were also commercially available products of A. R. grade.

Two kinds of biological tests were applied in the course of the isolation procedure to detect the presence of the biologically active component. One of them was the influence on the serum vitamin A level in rats published by Feuer et al.

* A large part of this paper has been presented at the Conference of the Hungarian Chemical Society, in 1977 (Furka et al., 1977).

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(1977). The other one based on the radioprotective effect was described by Feuer and Ormai (1978).

Gel filtration was carried out on Sephadex G-15 column. Equilibration and elution was performed with deionized water. In large scale preparations 5 g of protein free extract was fractionated on 140 × 4.3 cm column. V_0 and V_i was determined by passing through the column a mixture of ovalbumin and ammonium sulfate. Optical density was determined by Spectromom 203 (MOM, Hungary) spectrophotometer.

Ion exchange column was filled with Dowex 50 × 2 resin (H^+-form). For equilibration and elution pH 2 buffer was applied, the same which was also used for paper electrophoresis.

Paper electrophoresis was carried out on a flat-plate, water-cooled apparatus (Labor MIM, Hungary) at 31 V/cm. Buffers: pH 2, 20 ml of formic acid, 80 ml of acetic acid, 900 ml of water (Atfield, Morris, 1961); pH 3.5, 10 ml of pyridine, 100 ml of acetic acid, 890 ml of water (Ryle et al., 1955); pH 6.5, 100 ml of pyridine, 3 ml of acetic acid, 897 ml of water (Ryle et al., 1955). For electrophoretic and chromatographic separations Whatman 3MM paper was used. After drying, papers were examined under UV light (254 nm) then stained with the ninhydrin-cadmium reagent of Heilmann et al. (1957).

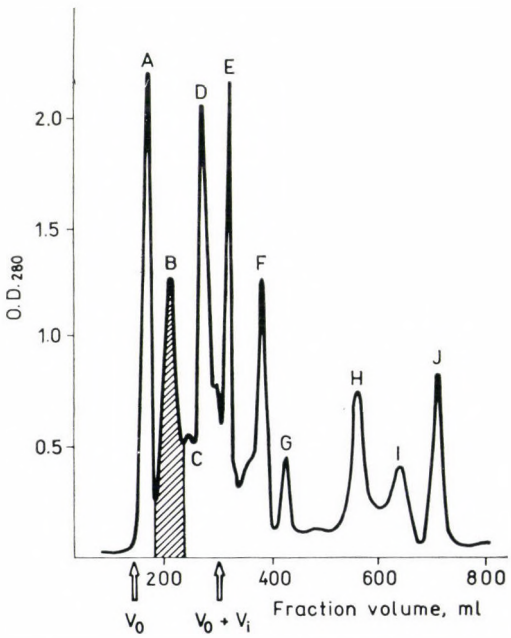


Fig. 1. Gel chromatography of the protein free extract of bovine parathyroid powder on Sephadex G15. The protein free extract (0.2 g) was eluted from the column (140 × 2.2 cm) with distilled water. Flow rate 18 ml/hour; fraction size 4.5 ml

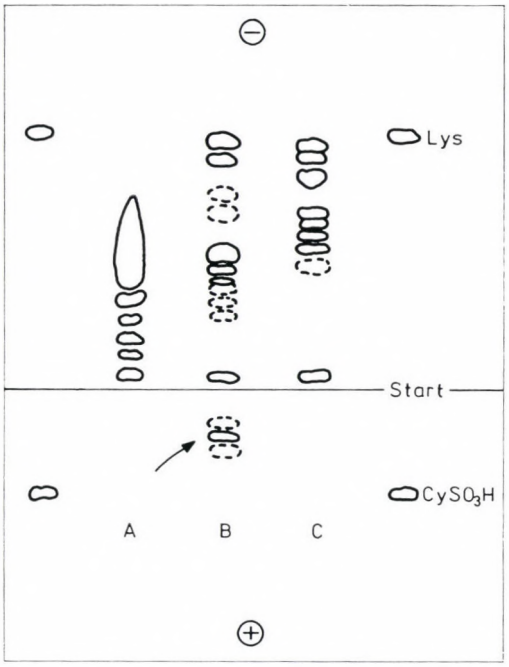


Fig. 2. Paper electrophoresis at pH 2 of fractions A, B and C. Load 0.25 mg/cm; time 90 minutes; 1500 V

Descending paper chromatography was applied in butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12, v/v; Light, Smith, 1962) for 60 hours.

Amino acid analysis was performed by a Hd 1200 E (Czechoslovakia) type analyzer after hydrolysis of the peptide sample in 6N hydrochloric acid for 20 hours.

Digestions by L-glutamic acid decarboxylase were carried out in pH 6.5 buffer in the presence of pyridoxal-5-phosphate for 2 hours as described by Roberts and Frankel (1951). The amino acids in the digests were identified by paper electrophoresis at pH 2.

Results and discussion

Gel chromatography

Samples of the protein free extract of bovine parathyroid powder were submitted to fractionation on Sephadex G15 column, using water as eluent (Fig. 1). Recovery based on the weight of the freeze dried fractions A–I was 90–95%. The fractions were submitted to biological tests. Both tests showed that the biological effect is associated with fraction B comprising 11% of the load.

Fractions A, B and C were examined by paper electrophoresis. The electrophoretic pattern obtained at pH 2 (Fig. 2) revealed that fraction B contains ninhydrine positive components (marked by arrow on Fig. 2) that are absent from the other two fractions. On the basis of this observation we assumed that the compound(s) responsible for the biological effect belong to these components, that move towards the anode even at pH 2.

Fractionation by ion exchange

At preparative paper electrophoresis at pH 2 the resolution of components of fraction B was not statisfactory. To facilitate separation of the strongly acidic components the electrophoretic step was preceded by fractionation on Dowex

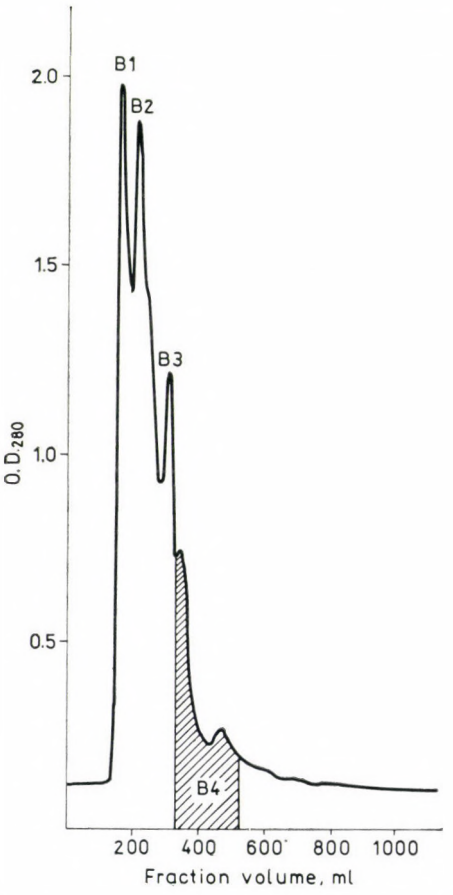


Fig. 3. Ion exchange chromatography of fraction B on Dowex 50×2. Load 0.9 g; column size 140×2.2 cm; eluent pH 2 buffer; flow rate 34 ml/hour; fraction size 21.4 ml

50×2 resin (Fig. 3). As detected by paper electrophoresis at pH 2, the strongly acidic substances responsible for the biological activity were accumulated in fraction B4. 0.9 g B yielded 76 mg of freeze dried B4.

Paper electrophoresis at pH 2

The electrophoretic pattern of B4 at pH 2 is shown in Fig. 4. The component B4C – sometimes appearing as a twin spot – was separated on preparative scale. The quantity of the component B4E detectable only under UV light as a dark spot was strongly reduced by the ion exchange treatment and this facilitated migration of B4C at heavier loads.

Paper chromatography of B4C

The shape of the B4C spot indicated the need for further separation. By means of paper chromatography B4C could be separated into four components (Fig. 5). The main components detectable by ninhydrin were B4C2 and B4C3. The other two spots were considered as impurities. B4C2 was prepared in larger quantities.

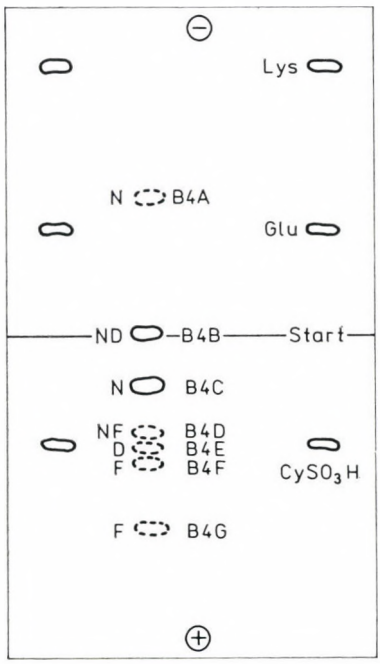


Fig. 4. Paper electrophoresis of B4 at pH 2. 1500 V; load 0.5 mg/cm; time 90 minutes. N: ninhydrin colour; D and F: dark and fluorescent in UV light

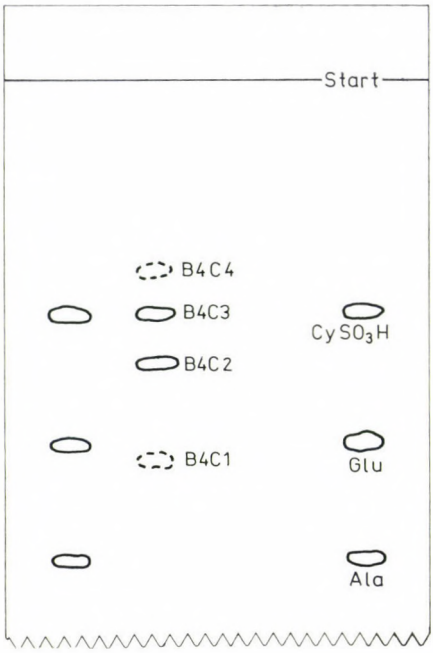


Fig. 5. Paper chromatography of B4C. Load 0.5 mg/cm; butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12); 60 hours

Purification by paper electrophoresis at pH 6.5

B4C2 as well as B4C3 was further purified by paper electrophoresis at pH 6.5 (Fig. 6). After this final step of purification the two samples were submitted to biological tests. Both tests gave the same result: B4C3 proved to be inactive, while B4C2 showed the same biological effects as did the protein free aqueous extract (doses by weight, protein free extract: B4C2 = 500 : 1). Fractionation of 150 g of protein free extract resulted in 5—10 mg of B4C2.

Composition of B4C2

Amino acid analysis of B4C2 indicated two components present in 1 : 1 molar ratio: glutamic acid and a component eluted on the analyser ahead of aspartic acid. This unknown component proved to be identical with taurine in the following experiments: elution on the amino acid analyser in the presence of added taurine, paper electrophoresis at pH 2 and pH 6.5 and paper chromatography.

In order to determine the number of glutamic acid and taurine residues present in the peptide molecule, a stock solution was prepared containing B4C2 and reference leucine in approximately the same concentration. Analysis of a sample of

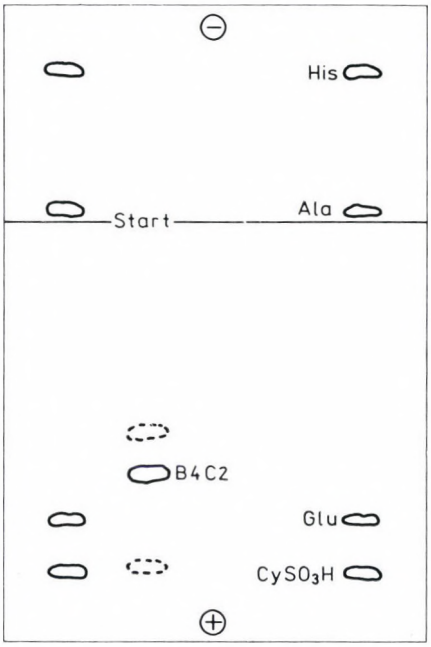


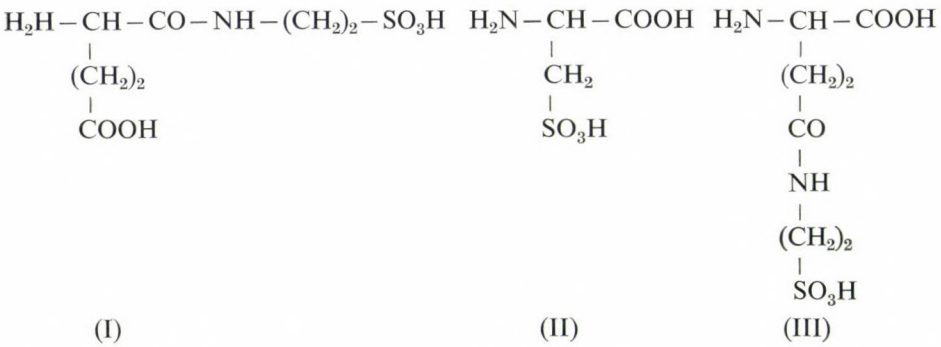
Fig. 6. Purification of B4C2 by paper electrophoresis at pH 6.5. 1500 V; 2 hours

this solution showed the molar ratio of B4C2 to leucine to be 1.28 : 1. A second sample was first submitted to acid hydrolysis and applied to the analyzer thereafter. The molar ratios of the formed amino acids to the reference leucine were: glutamic acid 1.30 : 1, taurine 1.13 : 1. Since the molar ratios of the liberated amino acids to leucine were not higher than that of the intact B4C2 to the reference substance, both glutamic acid and taurine were supposed to be represented in the molecule by a single residue each.

Configuration of glutamic acid formed on acid hydrolysis of B4C2 was determined enzymatically. A sample of B4C2 (100 nmol) was first hydrolyzed by acid then submitted to digestion by L-glutamic acid decarboxylase. As a result, the glutamic acid component of the hydrolyzate was decarboxylated to γ-aminobutyric acid. Thus, it can be concluded that the glutamic acid residue in B4C2 represents its L-enantiomer.

Structure of B4C2

Formulae (I) and (III) can be considered as possible structures for B4C2. The peptide can be stained with ninhydrin thus it is supposed to have a free amino group. Since it migrates towards the anode at pH 2 the molecule is expected to contain at least two strongly acidic groups.



(I) (II) (III)

One of them is the $-SO_3H$ group (pK 1.3 in taurine, Dawson et al., 1969) present in both (I) and (III). Taking into account the moderate acidity of the γ-carboxyl group (pK 4.25 in glutamic acid), the α-glutamyl structure (I) can be ruled out. The net charge of α-glutamyl-taurine at pH 2 would be zero. In γ-glutamyl structure (III), on the other hand, the strongly acidic α-carboxyl group (pK 2.19 in glutamic acid) is free and therefore the molecule is expected to carry a partial negative net charge even at pH 2. As far as the number and the type of the ionizable groups are concerned, structure (III) shows a close analogy to cysteic acid (II). Based on this analogy, changes in the pH of the buffer may be expected to bring about similar changes in the electrophoretic mobility of B4C2 and cysteic acid. As illustrated in Fig. 7, this was really found further supporting structure (III).

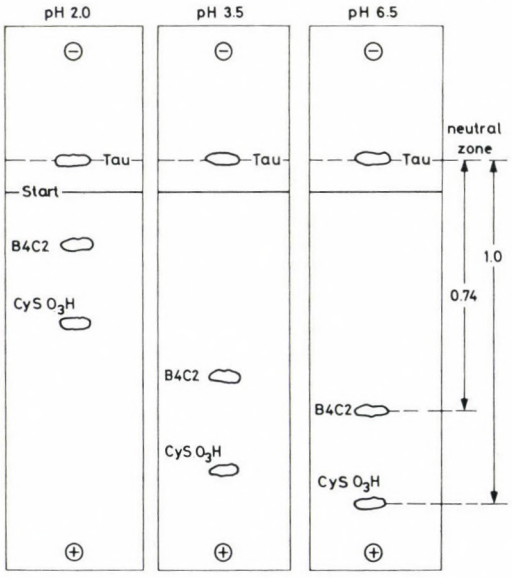


Fig. 7. Paper electrophoretic mobility of B4C2 compared to that of cysteic acid at pH 2, pH 3.5 and pH 6.5. Relative mobility at pH 6.5 is 0.74

From the relative mobility to cysteic acid (Fig. 7), a molecular weight of 265 was obtained for B4C2 (Offord, 1966), a value very close to 254.3 based on (III), indicating that there is no undetected constituent in the molecule.

The experimental evidences considered above show that B4C2 is γ-L-glutamyl-taurine, a dipeptide that has not yet been described in the literature.

Thanks are due to Chinoin Pharmaceutical Works Ltd., Budapest, for financial support.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 49–55 (1980)

Steroid Spectrum in Human Urine as Revealed by Gas Chromatography III. Identification and Quantitation of Pregnenediol at Different Stages of Bodily Development*

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(Received April 15, 1979)

Gas chromatographic analysis of steroids using Sp-2100 stationary phase revealed that all urine samples from 34 healthy children (aged 4 – 14 years) and from 35 adults contained 5-pregnene-3β,20α-diol, which was also identified by GC-MS. The extent of pregnenediol excretion continuously increased from prepuberty to adult age. The observed increase in pregnenediol excretion during prepuberty points to an enhanced biosynthetic activity of the adrenal gland in the years preceding sexual maturation.

Introduction

Besides steroid hormones, the adrenal cortex also secretes biosynthetic intermediates. Under physiological conditions, the amount of the steroid intermediates passing through the cell membrane and getting into the circulation is only a small fraction of the quantity of the biologically active hormones. The first steroid intermediate is pregnenolone; its secretion was inferred from the presence of pregnenediol in the urine of patients having adrenal cancer (Schiller et al., 1945; Hirschmann, Hirschmann, 1945) and also in that of healthy individuals (Wilson et al., 1961). The source of secretion was identified as the adrenal gland by Oertel et al. (1963) who demonstrated the arterio-venal concentration difference of pregnenolone.

Abbreviations: A, 3α-hydroxy-5α-androstan-17-one (androsterone); Ch, 5-cholesten-3β-o1 (cholesterol); DHA, 3β-hydroxy-5-androsten-17-one; E, 3α-hydroxy-5β-androstan-17-one (etiocholanolone); FID, flame ionization detector; GC-MS, gas chromatography combined with mass spectrometry; I, Kováts' retention index; 11-OE, 3α-hydroxy-5β-androstane-11,17-dione (11-keto-etiocholanolone); 11-OHA, 3α,11β-dihydroxy-5α-androstane-17-one (11-hydroxy-androsterone); 17-OH Pg, 3α, 17α-dihydroxy-5β-pregnan-20-one; Pd, 5β-pregnane-3α, 20α-diol + 5α-pregnane-3α, 20α-diol (pregnanediols); Δ5-Pd, 5-pregnene-3β, 20α-diol (pregnenediol); RRT, relative retention time; SE-30, methyl-substituted polysiloxan, stationary phase; SEM, standard error of the mean; Sp 2100, methyl-substituted polysiloxan, stationary phase.

* Supported by the Scientific Research Council, Ministry of Health, Hungary (3-13-0302-01-2/K)

After the introduction of radioimmunoassay-methods, the level of non-conjugated pregnenolone in peripheral blood was also determined (Bermundez et al., 1970). However, further possibilities for the determination of pregnenediol have not been sought, although the excretion of this substance gives information about the secretion of not only pregnenolone but also pregnenolone sulphate. In the present study we identified pregnenediol by the GC–MS method in our multicomponent urine steroid analysis; furthermore we studied the tendency of steroid excretion in the developing organism as compared to the pregnenediol value of adults.

Materials and methods

Reference steroids: 5-pregnene-3β,20α-diol and all the other reference steroids used were kindly supplied by Prof. D. N. Kirk (British Medical Council, Steroid Reference Collection, London).

Material studied: 24 hours' or 12 hours' urine was collected from 13 healthy boys and 21 girls, aged 4–14 years and from 12 men (20–50 years) and 23 women (18–38 years). When urine was collected for 12 hours, pregnenediol excretion was extrapolated to 24 hours.

Analytical methods: Acid hydrolysis of urine, toluene extraction of the steroids, their purification, preparation of acetyl derivatives and gas chromatographic analysis (in a Packard 7300 instrument) were carried out as described earlier (Kecskés, Juricskay, 1975) with the following modification: instead of 3% SE-30 as stationary phase, the column was packed with the industrial product of Supelco Inc. (Bellefonte), the composition of which was 3% SP-2100 stationary phase on Supelcoport 80/100 mesh support.

GC–MS measurements of the acetyl derivatives were also carried out by using the above stationary phase for a Pye-104 gas chromatograph connected to a Micromass MM 12FIA type magnetic mass spectrometer. The temperature of the column was 250 °C, the charrier gas flow (He) was 20 ml/min. The mass spectra were recorded using an ion accelerating voltage of 3 kV, an ionizing voltage of 20 eV, and an electron current of 20 μA. The temperature of the ionization chamber was kept at 200 °C.

Results

Fig. 1 shows the steroid spectrum of the urine of a 9-year-old girl. Steroid components were identified on the basis of the relative retention time to 5α-cholestane and retention index. The data of the identified components were identical with the retention values of the reference steroids. The pregnanediol peak (relative retention time 1.26) was followed by a peak marked "X_1" on the chromatogram (relative retention time 1.38; I_{250}^{Sp2100} : 2877), that had not yet been identified in our previous study. Component "X_1" was found to be present also in the urine of children; its quantity, however, was lower than in adults.

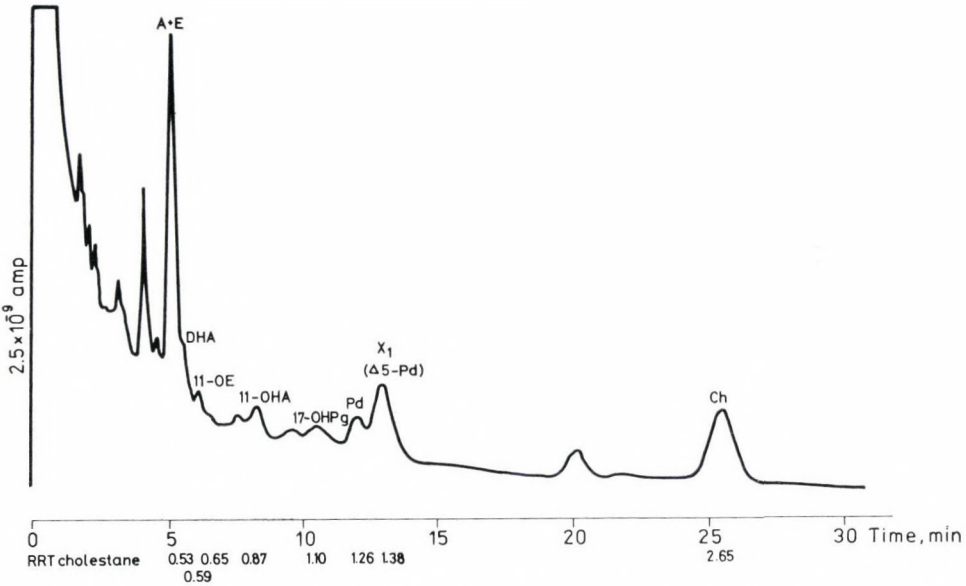

Fig. 1. Gas-liquid chromatogram of the acetyl derivatives of the urine steroids of a 9-year-old girl. Conditions: 3% Sp 2100 column, inlet temp.: 270 °C, column temp.: 250 °C, detection: FID

The first indication as to the nature of component "X_1" was obtained from the elution order. On a stationary phase of the methyl-silicone type the size of the component molecule can be inferred from the elution order if there is an identified component in its vicinity. Since peak "X_1" was eluted after pregnanediol, it seemed probable that the peak corresponded to a pregnanediol isomer. The retention values of the peak were identical with those of both 5α-pregnane-3β,20β-diol-diacetate and pregnenediol-diacetate. Because of the relatively small amount of material, we did not make attempts to isolate the unknown component by preparative gas chromatography but carried out GC–MS measurements for some of the urinary extracts. In each of these cases, the material in peak "X_1" yielded the same mass spectrum (Fig. 2A). No molecular ion corresponding to 5α-pregnane-3β,20β-diol-diacetate, or to that of pregnenediol-diacetate (m/e 404 and 402, respectively) could be observed in the mass spectrum. The formation of the ion with the highest mass number detected at m/e 342, can be explained by the elimination of one molecule of acetic acid from pregnenediol-diacetate.

The mass spectrum of 5-pregnene-3β,20α-diol-diacetate, recorded under the same conditions (Fig. 2B) was found to be identical with that of the material in peak "X_1". No molecular ion could be observed here again, and the first intensive ion peak is the m/e 342 peak, formed by the loss of an acetic acid molecule. From the two alternatives offered by the gas chromatographic retention values, the

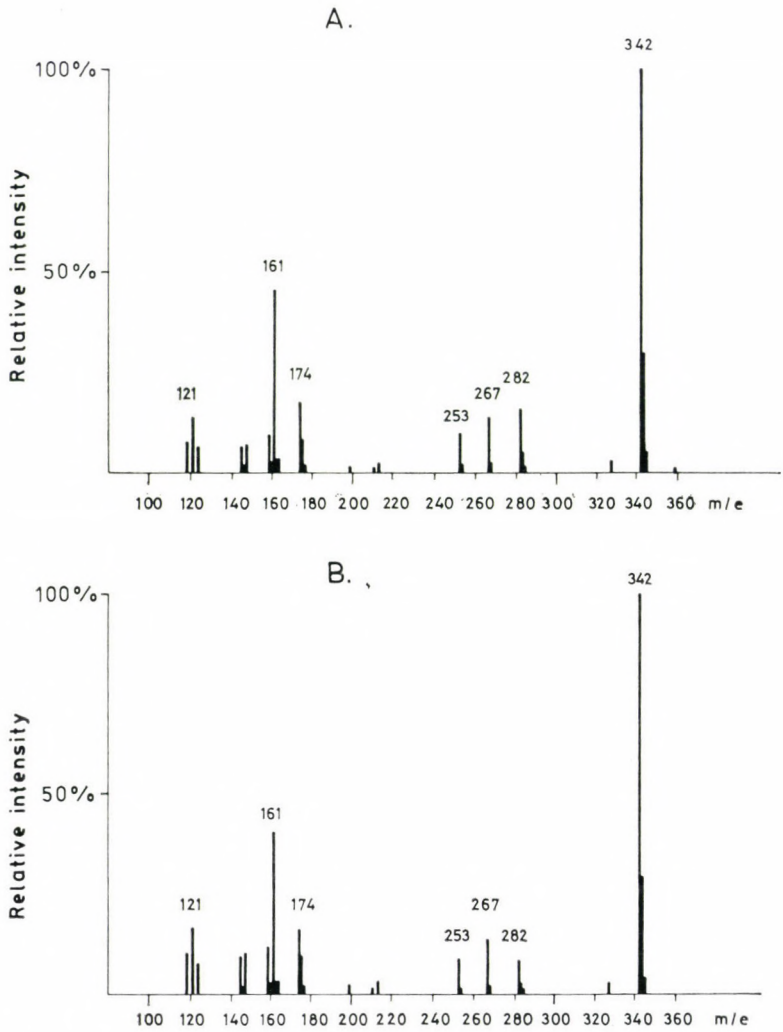

Fig. 2. Mass spectrum of the acetyl derivative of component "X_1" (A) and 5-pregnene-3β,20α-diol (B)

material in the unknown peak was thus identified as pregnenediol by mass spectrometry and found to be homogeneous.

The results of a quantitative analysis are presented in Fig. 3. The mean values for pregnenediol excretion characteristic of the individual age groups, expressed in μg/day $\pm$ SEM were the following: 4–7-year-old children (2 boys, 9 girls) 35.8 $\pm$ 9.2; prepubertal group (9 boys, 7 girls) 93.0 $\pm$ 23.4; pubertal group (2 boys, 5 girls) 152.6 $\pm$ 33.4; adult men (12 individuals) 315 $\pm$ 60.0; adult women (23 individuals) 450 $\pm$ 45.3. Pregnenediol could be traced from the age of 4, and its

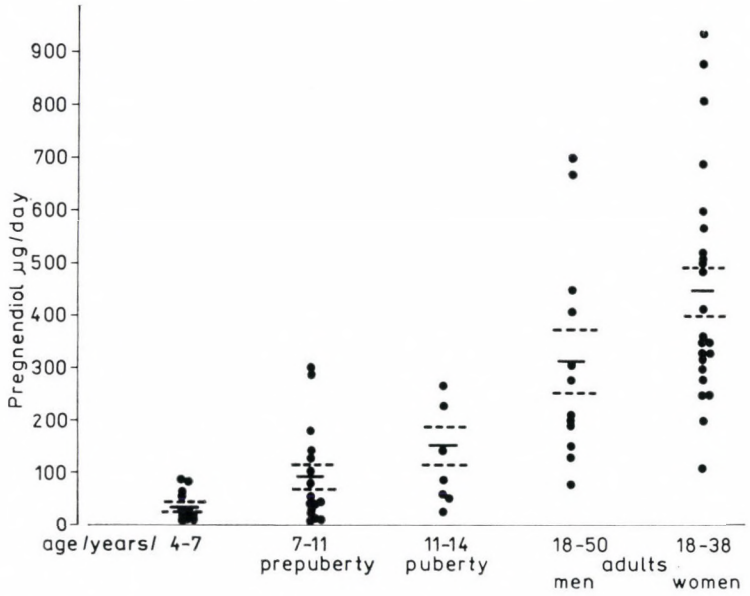

Fig. 3. Pregnenediol excretion in the urine of children of different ages and in that of adults, mean ± SEM

amount continuously increased from prepuberty to adult age. By means of the t-test, the mean value for children aged 4–7 years and that for children in prepuberty were shown to be significantly different ($p < 0.05$).

For adults a sexual difference in pregnenediol excretion was established in favour of women; however, this difference was not significant ($p > 0.05$).

Discussion

In our experiments, pregnenediol excretion was evidenced also for 4–7-year-old children and its extent was found to increase significantly in prepuberty. It is well known that also the serum level of DHA starts to increase at this developmental stage. Several authors consider the latter increase as the first sign of prepuberty and suggest that an increase in androgen secretion of the adrenal gland plays a role in processes leading to puberty (Sizonenko et al., 1975; Ducharme et al., 1976). Apter and Vihko (1977) studied the pregnenolone level in the plasma from the age of 7 and found a significant increase in different stages of puberty. Increase in pregnenolone level was found to be parallel with that in DHA level during puberty.

We observed a further increase in pregnenediol excretion in puberty as compared to prepuberty and this increasing tendency was continuous until adult

age. We attribute the increase in pregnenediol excretion after puberty to pregnenolone secretion of gonadic origin. Pregnenolone secretion by the gonads was proved for men on the basis of the concentration difference between the vena spermatica and the cubital vein by Hammond et al. (1977) and for women, after bilateral adrenalectomy by determinations from the cubital vein by Abraham and Chakmakjian (1973).

We found pregnenediol excretion at adult age to be higher in women than in men. Since the time of collecting urine from females always fell at the time of the luteal maximum of the menstruation cycle and, with the exception of 3 cases, the amount of pregnanediol always indicated the presence of the corpus luteum, one could suggest that the corpus luteum also contributed to the higher pregnenediol excretion. However, data in the literature on pregnenolone secretion by the corpus luteum are contradictory. Bermundez et al. (1970) and Abraham et al. (1973) found the pregnenolone level to be higher in the luteal phase than either in the follicular phase or in men. DiPietro et al. (1972) and McKenna and Brown (1974) found no difference between the two phases of the cycle, however, the number of cases in the luteal phase studied by the latter authors was low. Opinions thus differ and therefore the question of pregnenolone secretion by the corpus luteum is far from being settled.

A comparison of our observations with the results of Apter and Vihko (1977) suggests that the significant prepubertal increase in pregnenediol excretion indicates an increasing activity of the adrenal gland in the years preceding sexual maturation.

The role of the adrenal gland in the processes leading to puberty is not clear yet. Several authors suggest that DHA has a senzitizing effect in the maturation process of the hypothalamus-hypophysis-gonad system (Sizonenko et al., 1975; Ducharme et al., 1976). The intrinsic biological effect of pregnenolone in this respect is unclear. However, several authors have reported the interaction of pregnenolone with other steroids, observed during *in vitro* studies on endocrine organs. In bovine adrenal gland, 17-hydroxy-progesterone was found to inhibit the pregnenolone-progesterone conversion (Patwardhan, Lanthier, 1971), while in the corpus luteum the same conversion was shown to be inhibited by DHA (Dorfman et al., 1964).

It is an open question whether or not the prepubertal increase in pregnenediol excretion is affected by similar mechanisms at the adrenal level. In order to clarify the possibilities of *in vivo* regulation, further investigations are needed.

Thanks are due to Prof. D. N. Kirk (Steroid Reference Collection, London) for making the reference steroids available for us and to Dr J. Tamás (Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest) for his help in carrying out the GC-MS measurements and in evaluating the mass spectra.

The enthusiastic technical assistance of Mrs Zsuzsanna Sándorfi and Mrs Éva Deák is gratefully acknowledged.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 57—63 (1980)

Effect of Antibiotics Influencing Membrane Function on the Potassium Transport of *E. coli* Cells

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(Received May 18, 1979)

The effect of polymixin, nigericin, gramicidin on the ^{42}K-efflux of *E. coli* cells was studied. The ^{42}K-efflux of the bacteria decreases in time according to an exponential function. The slopes of the linearized functions characterizing the efflux increase with increasing concentration of antibiotics. The frequency of events of the ^{42}K-release as a parameter of antibiotics membrane interaction was determined on the basis of a theoretical model developed for the evaluation of our experimental data. In this way a quantitative comparison of the effectiveness of antibiotics was possible. We found, that the most effective antibiotic was polymixin, followed by nigericin and gramicidin.

Introduction

The fact that the antibiotics influencing membrane function accelerate the fluxes of cations through the membrane plays a significant role in their biological activity (Harold, Baarda, 1968; Gale, 1973; Pressman, 1973; Mc Laughlin, Eisenberg, 1975). The change in membrane permeability can be demonstrated already at the early period of antibiotic action. (Imai et al. 1975; Chuen-Chin et al. 1973). Therefore the measurement of cation fluxes is a sensitive indicator of the interaction between antibiotics and membranes and that of the effect exerted on the structural integrity of the membrane (Silver, Bhattacharya, 1974; Gale et al 1972; Kaback, 1972). In our previous experiments the effect of streptomycin on the ^{42}K-release from *E. coli* cells was investigated (Tamás et al. 1971). In this paper all results concerning the effect of three other antibiotics (polymixin, nigericin, gramicidin) on the ^{42}K-efflux of E. coli cells are presented. The number of antibiotic molecules adsorbed on the bacterial surface and the change in membrane permeability was compared on the basis of our previous results. (Szőgyi et al. 1977.)

Methods

An *E. coli* B strain was used in our experiments. The preparation of the bacterium culture for the experiments was described elswhere (Szőgyi et al. 1977). The bacterial cells were incubated for 30 minutes in bouillon medium containing 300 μCi of ^{42}KCl. The radioactive medium was removed by centrifugation and after a quick washing with growth medium the cells were resuspended in nonradio-

active medium. A part of the suspension was used as a control, that was diluted to $\sim 10^8$ bacteria/ml. The other samples were diluted by medium containing antibiotics to the same volume as the control one. The concentration of the antibiotics in the samples varied between 10–40 μg/ml. To determine the ^{42}K-content and the number of viable bacteria samples were taken at given time periods. The samples were filtered on G-5 filter. The bacteria remained on the filter were washed three times with nonradioactive medium and the ^{42}K-activity of the cells was measured.

Our experiments were carried out under such conditions (concentrations of antibiotics, incubation time) that there was no detectable change in the number of viable bacteria, in this way we could investigate only the membrane permeability increasing effect of antibiotics.

Polymixin B sulphate (Pffizer Co. Inc. USA), Gramicidin (Koch-Light Labor Columbrook, England), and Nigericin (Commercial Solvents Corp. Terre Haute USA) were used in our experiments.

Results

The results of our experiment with polymixin are shown in Fig. 1, where the decrease of the specific ^{42}K-activity of bacteria is plotted against the incubation time in a semilogarithmic coordinate system. The uppermost straight line (K) was

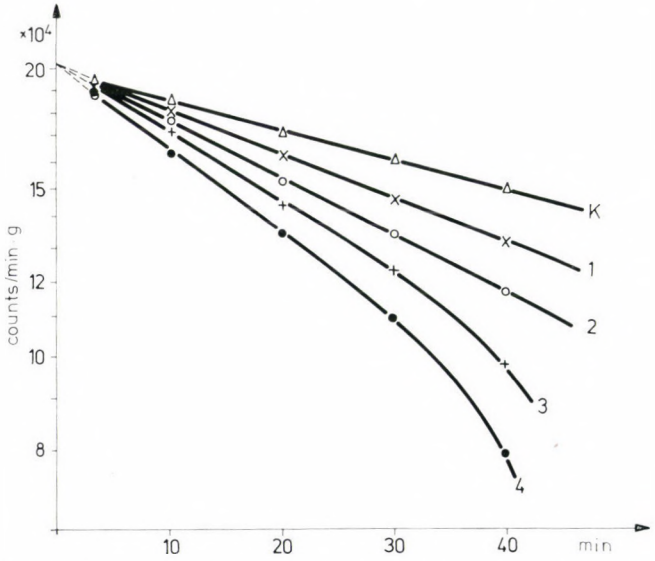

Fig. 1. Effect of polymixin on the ^{42}K-efflux of *E. coli* cells. The straight line (K) shows the ^{42}K-efflux in the case of a medium containing no antibiotics: the line denoted with 1 — represents the experiment with a medium containing polymixin at a concentration of 10 μg/ml; line 2 — polymixin concentration of 20 μg/ml; line 3 — concentration of 30μg/ml; line 4 — concentration of 40 μg/ml. Each point means the average of 6 to 8 experiments.

obtained under normal conditions – the medium did not contain antibiotics –, the others (1–4) belong to 10, 20, 30, 40 μg/ml polymixin concentrations. The line K in the Fig. 1. shows that the ^{42}K-activity of the intact bacteria decreases accord-

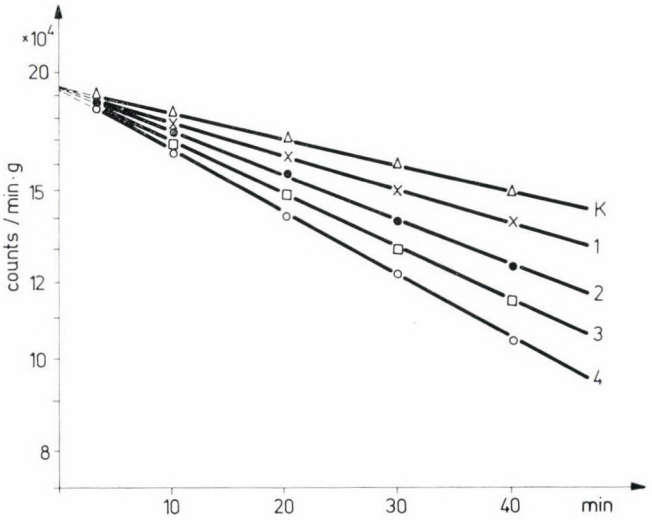

Fig. 2. Effect of nigericin on the ^{42}K-efflux of *E. coli* cells. The straight line (K) shows the ^{42}K-efflux of the control culture; line 1 shows the ^{42}K-efflux in the case of a medium with a nigericin concentration of 10 μg/ml; line 2 – in the case of a concentration of 20 μg/ml; line 3 – concentration of 30 μg/ml; line 4 – concentration of 40 μg/ml. Each point means the average of 6 to 8 experiments.

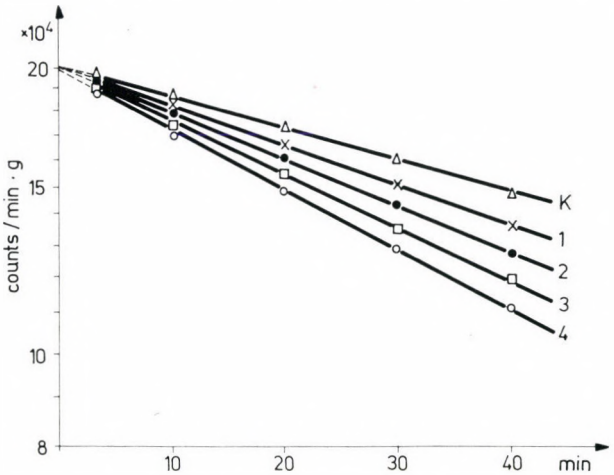

Fig. 3. Effect of gramicidin on the ^{42}K-efflux of *E. coli* cells. The straight line (K) shows the ^{42}K-efflux in the case of a medium not containing antibiotics, line 1 – gramicidin concentration of 25 μg/ml, line 2 – concentration of 50 μg/ml; line 3 – 75 μg/ml, and line 4 – concentration of 100 μg/ml. Each point shows the average of 6 to 8 experiments.

ing to an exponential function. The decrease of ^{42}K-activity of bacteria in the presence of antibiotics is also exponential, the slope of the straight lines increases with the increase of polymixin concentration. At higher antibiotic concentrations (30 and 40 μg/ml) and at longer incubation times (30–40 min.) however there is a deviation from the exponential character, the ^{42}K-efflux is higher than it can be expected on the basis of the exponential function. This phenomenon can be explained by the fact that the binding of polymixin to the bacterial membrane causes such a structural change, that the membrane is no more able to act as a barrier.

Fig. 2. shows our experimental results referring to the effect of nigericin on the ^{42}K-efflux of bacteria. It can be seen that similarly to the data obtained with polymixin, the potassium efflux increases with the increase of nigericin concentrations (10–40 μg/ml), but the deviation from the exponential function observed at the application of polymixin at higher concentrations was not established at higher concentrations of nigericin.

Fig. 3. shows the ^{42}K-efflux of E. coli cells in the presence of gramicidin. To get a measurable change in the potassium efflux of bacteria the gramicidin had to be applied in higher concentrations than that of the other antibiotics (25, 50, 75, 100 μg/ml).

Discussion

The experimental results are interpreted in a similar way as the effect of streptomycin on the ^{42}K-efflux was discussed in our previous paper (Tamás et al. 1971.)

α denotes the proportion of the bacterium surface influenced by the antibiotic molecules, i.e.

$$\alpha = \frac{N}{N_0} \tag{1}$$

where N_0 means the average number of regions on the surface of bacteria which can be influenced by antibiotics, N means the average number of regions actually influenced by the adsorbed antibiotic molecules. It is obvious that the intact proportion of the bacterial surface is $(1 - \alpha)$. According to our theoretical model in the presence of antibiotics two factors determine the decrease of ^{42}K-content of bacteria. The probability of the release of a potassium ion from the cell is equal to $(1 - \alpha)\, k\mathrm{d}t$ in the regions of membrane not influenced by the antibiotic molecules and it is equal to $\alpha\, k_a \mathrm{d}t$ in the influenced regions. The values of k and k_a denote the frequency of events of potassium release in the case of intact and influenced membrane resp. The change of the ^{42}K-activity of one bacterium can be described with following equations:

$$-\frac{\mathrm{d}n}{n} = (1 - \alpha)\, k\mathrm{d}t + \alpha k_a\, \mathrm{d}t\,, \tag{2a}$$

and $$n = n_0 \exp\{-[k + (k_a - k)\alpha]t\}, \quad (2b)$$

where n_0 and n denote the average number of ^{42}K-atoms in one bacterium at the beginning of the measurement (t = 0) and at *t* incubation time resp. The equation 2b correlates well with our experimental data showing an exponential function between the ^{42}K-efflux and the incubation time at the antibiotic concentrations investigated. In the equation 2b. the values of n and n_0 can be replaced by the appropriate specific activities. In this way the slope of the linearized exponential function is equal to $k + (k_a - k)\alpha$ and can be calculated from the experimental data. The three components of the slope (k, k_a, α) can be separately calculated because the *k* value can be determined from the exponential function describing the ^{42}K-efflux of the control samples and the α value depending on the quality and concentration of the antibiotics can be measured experimentally (Szőgyi et al. 1977). So k_a can be calculated if the value of the expression is known.

Our results of theoretical interest are summarized in Tables 1–3. The first column of the tables contains the concentrations of antibiotics (c), the second the degree of influence (α), the third and the fourth show the data which were determined from the slope of the linearized exponential functions. Tables 1, 2, 3.

Table 1

Data characterizing the effect of polymixin influencing ^{42}K-efflux

1	2	3	4
C $\mu g(ml)^{-1}$	$10^{-1}\alpha$	$10^5 (k_a - k)\alpha$ s^{-1}	$10^4 (k_a - k)$ s^{-1}
10	2.5	6.8	2.7
20	4.5	12.3	2.7
30	6.1	18.0	2.9
40	7.1	22.2	3.1

Table 2

Evaluation of the effect of nigericin influencing ^{42}K-efflux

1	2	3	4
C $\mu g(ml)^{-1}$	$10^2\alpha$	$10^5 (k_a - k)\alpha$ s^{-1}	$10^3 (k_a - k)$ s^{-1}
10	1.9	5.5	2.8
20	3.8	10.0	2.7
30	5.4	15.0	2.8
40	7.1	20.0	2.8

Table 3

Data characterizing the effect of gramicidin influencing the ^{42}K-efflux

1	2	3	4
C $\mu g(ml)^{-1}$	$10^{-2}\alpha$	$10^5 (k_a - k)\alpha$ s^{-1}	$10^3 (k_a - k)$ s^{-1}
25	1.2	3.3	2.8
50	2.3	6.5	2.8
75	3.4	9.5	2.8
100	4.5	12.5	2.8

From the data the following theoretical conclusions can be drawn:

a) the expression $(k_a - k)\alpha$ shows to what extent the frequency of events of ^{42}K-efflux increases as a consequence of the interaction between the antibiotics and the membrane in the case of one bacterium. This expression can be considered as a parameter which characterizes the effectiveness of different antibiotics in the case of a given bacterium. According to our data the polymixin is the most effective of the three antibiotics investigated and it is followed by nigericin and gramicidin. The effectivity of streptomycin is between nigericin and gramicidin according to our previous investigations.

b) the value of $(k_a - k)$ shows the change in frequency of events of potassium efflux for one bacterium, when the whole bacterium membrane is influenced by antibiotics ($\alpha = 1$). Assuming that a bacterium membrane has N_0 influencable regions, the value $(k_a - k)/N_0$ gives the change in frequency of events of efflux for one region. As it can be seen in the fourth columns of the Tables 1–3, the values $(k_a - k)$ are nearly constant for all the three antibiotics. We obtained an approximately same $(k_a - k)$ value for streptomycin in our previous experiments. As the value N_0 can be considered as a parameter characterizing the bacteria, the expression $(k_a - k)/N_0$ is nearly constant too in the case of the antibiotics investigated. This fact indicates, that under the given conditions these antibiotics cause a similar membrane damage from the point of view of potassium efflux. This conclusion is in accordance with the suggestion of Hartman et al (1978), that the damage can be explained by a structural rearrangement which takes place in the membrane in consequence of the interaction between the antibiotics and the membrane components.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 65—71 (1980)

Chromatographic Study of the UV Photoproducts in Uracil Solid Phase Systems

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(Received April 14, 1979)

UV photoproducts were induced in two types of uracil solid phase systems: in evaporated and recrystallized thin layers and in dried aquaeous solution respectively. The nature of the photochemical reactions in the two systems was compared by gradient elution column chromatography. In the case of dried solution two types of cyclobutane dimers were isolated, but in recrystallized thin layers only one type was found. The efficiency of the reaction in evaporated layers was much higher than in those produced by drying. Based on the crystal structure of uracil and previous absorption spectroscopic studies of the photochemistry of thin crystalline layers it was stated, that the single type of photodimers produced in evaporated thin layers is characteristic for the photochemical reaction proceeding in crystal matrix; and its structure was proposed to be identical with that of the cis-syn stereoisomer dimer.

Introduction

It is a well-known fact, that the UV radiation injury of native RNA originates mainly from the UV photochemical changes of its uracil bases (Fidy, Karczag, 1974; Karczag et al., 1973; Remsen et al., 1971). One of the UV photochemical processes under consideration is the cyclobutane-type photodimerization of uracil in the cis-syn stereoisomer structure (Adman et al., 1968) thus the understanding of this phenomenon would evidently be of biological importance.

Usually by the study of some model systems of a complex phenomenon it is possible to reduce the complexity of the systems under consideration. In the case of the radiation processes in RNA such a model system which would involve only a single photochemical reaction was not found. All of the studied model systems were too complex for the understanding of any of the processes separately (Brown, Johns, 1968; Jennings et al., 1972; Pearson, Johns, 1966; Varghese, 1971).

Our previous investigations (Fidy, Raksányi, 1978) suggested, that it would be possible to produce highly crystallized microcrystalline thin layers of uracil by vacuum evaporation in which the UV photochemical reaction takes place in the crystalline regions of the sample. This assumption was based on an absorption spectroscopic study of the layers, and the quantum efficiency of the photochemical conversion of uracil was determined. The presence of the crystalline matrix means

given topochemical factors for the photochemical reaction. If the topological conditions fulfil the requirements prescribed by one of the possible reaction paths, the dimerization follows this way even though it is not the most favourable in the absolute sense, i.e. corresponding to a gas-phase reaction. This way the crystal exerts a stereoselective effect on the conformers or other molecular species arising from a chemical reaction (Cohen, Schmidt, 1964). Thus it seems probable, that there is a single geometry which meets the most favourable conditions for dimerization in the crystal, and the spectroscopically determined conversion quantum efficiency characterizes the formation probability of this very photoproduct which was selected by the matrix. In the present work we aimed to examine whether there exists a single product in microcrystalline samples or the reaction is complex in this model system as well as in the systems used by others.

Materials and methods

Uracil (Reanal, Hungary) was used without any further purification. The purity of the powder-form material was checked by the comparison of the absorption spectrum of its aquaeous solution with that of literature data. Two types of solid phase systems were studied. One was produced by drying 100 drops of uracil solution. The volume of one drop was 0.01 ml and contained 10^{-5} gr of uracil. The series of dried drops was irradiated at $\lambda = 254$ nm with a 15 W low pressure mercury lamp (General Electric) for 4 hours. The other system was produced by vacuum evaporation of the uracil powder. 30 plate-like thin layers were formed on quartz substrates of a diameter of 1.6 cm. The amount of uracil in one thin layer was about 4×10^{-6} g with an extinction of about 0.5 at 254 nm. Each layer was aged at 70 °C and was left lying in air for two days. During this procedure an absorption-measurement-controlled recrystallization took place in the layers (Fidy, Raksányi, 1978; Gumenyuk et al., 1976; Sinsheimer et al., 1949; Yamada, Fukutome, 1968). The recrystallized layers were irradiated with the same source for 1 minute. Both types of irradiated samples were collected by being dissolved in distilled water. The quality of the samples was determined by absorption controlled gradient elution column chromatography. The scheme of the experiment is seen in Fig. 1. An anionic exchange column of Dowex 1×8 resin, 130×7 mm was made, which was washed for several days with 1 N formic acid and 1 N NH_4OH before use. The elution was performed with 0.1 N HCl-0.1 N NH_4OH buffer of continuously changing pH. The pH of the column before a run was fixed by leaving the column overnight in 0.1 N HCl, washing it with 0.1 N NH_4OH and finally eluting it with the content of the mixing chamber for two hours. When the sample was posited on the column, the pH of the mixing chamber (100 ml) was 9.8 and that of the reservoir 5.6 (see Fig. 1). The elution rate was 0.2 ml/min. To develop uracil from the column, after 6 hours an elution with 0.1 N HCl was applied. The absorption of the effluent at $\lambda = 200$, 210, 260, 300 nm resp. was continuously recorded by a Perkin Elmer 200 spectrophotometer using quartz

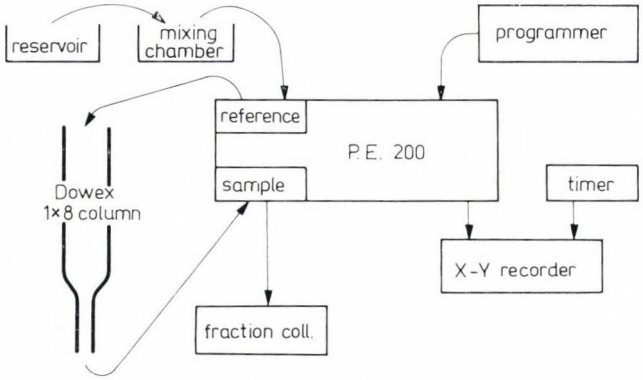

Fig. 1. The scheme of the gradient elution column chromatographic method we used. The elution rate was 0.2 ml/min

flow-cells. Fractions (3 ml) were collected automatically by an LKB 1700 Minirac fraction collector, and were analysed separately for pH and absorption spectra.

Results and discussion

Though the chromatography of nucleotide bases has already been studied, it is still an actual problem (Miller et al., 1976; Uematsu, Suhadolnik, 1976). Column chromatographic methods were applied for the separation of uracil and its UV photoproducts by Varghese (1971) and Jennings et al. (1970; 1972). Comparing these techniques with ours, slight differences can be found. In both of the cited works formic acid was used as acidic component. In our experiment this component was replaced by HCl to avoid the significant end-absorption of formic acid in the UV region of the spectrum. Jennings et al. (1970) applied a fast elution using 1 N formic acid and 1 N NH_4OH as reservoir and mixing chamber respectively. However, the use of these concentrated solutions is not too favourable because of the possible instability of the photoproduct in them, and the fast elution technique leads to the appearance of all dimeric species in the same fraction. The UV photochemistry of dried uracil layers was studied by Wang (Wang, 1961) and a single type of photodimers was reported as well as in the case of irradiated frozen aquaeous solution. However, in the work of Varghese cited above two types of cyclobutane photodimers were isolated from irradiated frozen solutions. The major product was found to be of the cis-syn type, the other was of cis-anti structure. Up to now evaporated and recrystallized thin layers have not been investigated.

Fig. 2a shows the elution profile of irradiated dried drops of uracil solution as determined in our experiment. The extinctions at $\lambda = 210$ nm (full line) and at $\lambda = 260$ nm (dashed line) are presented as functions of time, as they were recorded by the spectrophotometer. Two parallel runs were carried out. In both cases, two pronounced absorption peaks at $\lambda = 210$ nm could be seen at the early fractions,

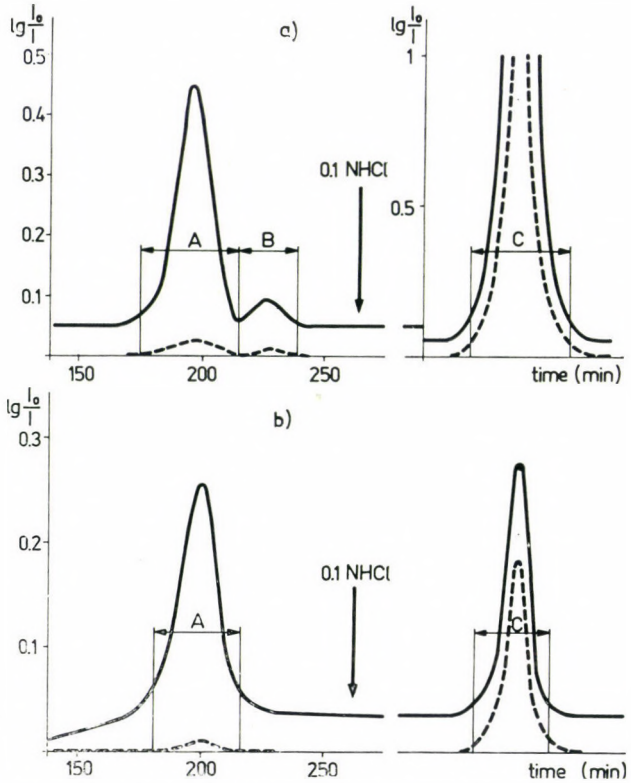

Fig. 2a. The gradient elution profile of uracil, irradiated in the form of dried drops. The extinction scale refers to $\lambda = 210$ nm (full line) and $\lambda = 260$ nm (dashed line) respectively
Fig. 2b. The gradient elution profile of uracil irradiated in the form of evaporated thin layers. The extinction scale refers to $\lambda = 210$ nm (full line) and $\lambda = 260$ nm (dashed line) respectively

and after and elution with 0.1 N HCl a third peak appeared. The distance and the relative height of the two early peaks was the same in the two parallel experiments. The pH change during this period was so small, that it did not exceed the standard error of its measurement, thus the exact position of the peaks versus pH could not be determined. The pH in the reference path before the column (Fig. 1) was about 9.1 when these two peaks developed. In the figure the extinction at $\lambda = 260$ nm can also be seen. The extinction at $\lambda = 200$ nm shows the same profile as that at $\lambda = 210$ nm, consequently it is not presented here. The extinction at $\lambda = 300$ nm was zero during the whole run except in the region of the third precipitate, where it ran parallel with the extinction at $\lambda = 260$ nm with a peak value of 0.03.

Fig. 2b shows the elution profile of the irradiated thin layers. In this case, the extinction at $\lambda = 210$ nm shows only one maximum in the early fractions, and a second one after elution with 0.1 N HCl. The position of the single early peak plotted against time was 200 min, practically the same as that of the first peak in the case of dried drops. The comparison of Figs. 2a and b clearly shows that the

reaction is much more effective in the case of evaporated thin layers. In irradiated solid systems produced by drying, the high light scattering and self-absorption of the layers reduce the effectivity of the reaction.

The fractions containing precipitates were tested for quality in each case. Fractions A and B (see Figs. 2a and b) just after being collected were brought to neutral pH by dilution with 0.1 N HCl. Then the solutions were irradiated with 254 nm UV light for 1 hour. In Figs. 3a and b the changes in the absorption spectra can be seen. Originally neither of the fractions exhibited absorption above 240 nm, but after irradiation a maximum at $\lambda = 260$ nm appeared (dashed lines). This is a well known test for cyclobutane-type photodimers of the pyrimidine bases (Fisher, Johns, 1976) which can be converted to monomers by irradiation with UV light. The spectra of the solutions in their original state were consistent with those of the cyclobutane dimers (Smietanowska, Shugar, 1961; Swenson, Setlow, 1963) and after irradiation the band position was identical with that of the spectrum of uracil at neutral pH. The absorption spectrum of fraction C (See Figs. 2a and b) was

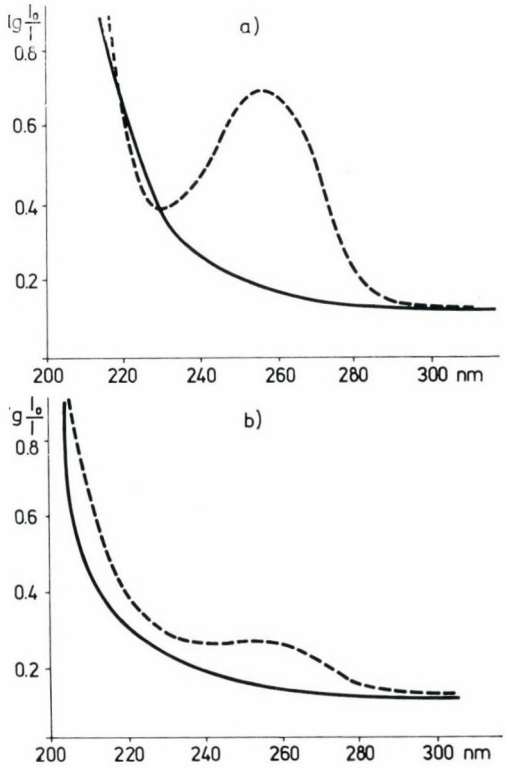

Fig. 3a. The absorption spectrum of the chromatographically isolated fraction A — in original state (full line) and after irradiation with UV light for 1 hour (dashed line)

Fig. 3b. The absorption spectrum of the chromatographically isolated fraction B — in original state (full line) and after irradiation with UV light for 1 hour (dashed line)

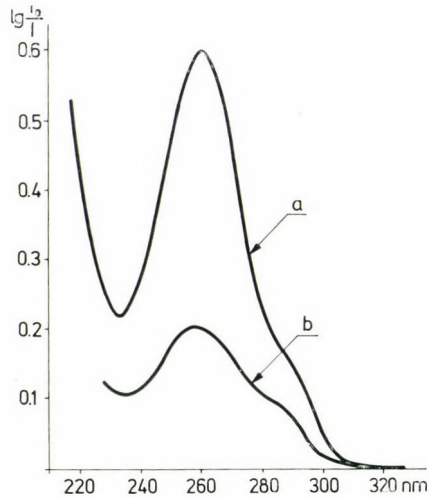

Fig. 4. The absorption spectrum of 10^{-4} M uracil solution at pH 9 — curve *a;* and that of the chromatographically isolated fraction C — curve *b*

identical with that of uracil at slightly basic pH (Fig. 4). The fractions A and B were tested for stability; they were left at basic pH and were boiled at acidic pH. In each case we got some conversion to uracil. The relative amount of the converted part, however, could not be determined, because the original amount of the precipitate was not known. Thus on the basis of the stability differences of the various types of cyclobutane dimers (Jennings et al., 1972; Varghese, 1971) we could not identify the isomer structure characterizing the fractions.

Our experimental findings showed that in the case of dried drops of uracil solutions the UV photochemical changes resulted in the formation of two types of photodimers but in recrystallized thin layers only a single type of cyclobutane dimers was formed. The photochemical conversion was much more effective in the case of recrystallized layers (Fidy, Raksányi, 1978). Comparing the elution profile of dried drops with that of frozen aquaeous solutions of uracil reported by Varghese (Varghese, 1971) the similarity in the distance of the dimer fractions and in their relative height is remarkable. If we accept Wang's argument (Wang, 1961) that there is no significant structure difference between dried layer and frozen solution we may assume that the major product from dried drops of uracil is the same as that from frozen solutions: the cis-syn type cyclobutane dimer. If this is true, this very structure characterizes also that single photodimer which was detected in recrystallized microcrystalline layers. Considering the crystal structure of uracil (Stewart, Jensen, 1967) in the crystal matrix one also would expect to obtain the cis-syn structure (Adman et al., 1968) of the possible photoproducts. This conclusion coincides with our previous findings (Fidy, Raksányi, 1978), that in thin recrystallized layers at the early stages up to about 60 per cent conversion, the photochemical reaction proceeds in the crystalline regions of the sample. Naturally

if we apply higher doses of irradiation, the reaction will run through the microcrystals and will be driven to the adjacent amorphous regions where the topochemical postulate fails to be valid. Thus in this case the reaction may lead to various types of photoproducts.

Summarizing our results we can say that in evaporated thin layers of uracil – using appropriate doses of irradiation – the effective production of a single type of photodimers may be achieved. As the system is suitable for absorption spectroscopy, a single reaction can be followed in situ and thus quantitatively characterized by the registration of the spectra (Fidy, Raksányi, 1978; Dobos et al., 1979).

The authors would like to express their thanks to Dr Gy. Rontó and prof. I. Tarján for their continuous help and interest. The technical assistance of K. Tárkányi, L. Ötsi and L. Pálhegyi is highly appreciated.

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[1] This work was presented as a poster on the 12-th FEBS Meeting, Dresden, 1978.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (1), pp. 73—75 (1980)

On the Phase Diagram of the Dipalmitoyl Phosphatidylcholine Single-bilayer

Comparison with the multilamellar system

(Short Communication)

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(Received May 10, 1979)

Though single bilayers have certain biological importance, the structure of multilamellar systems have been studied more extensively, for technical reasons. Therefore it is important to focus our attention on the differences between the properties of these systems. The phase diagram of the dipalmitoyl phosphatidylcoline single-bilayer is constructed by means of available experimental data, of simple considerations on the interfacial water structure and of reasonable analogies on the phase diagram of multilayers.

Janiak et al. (1976), Ulmius et al. (1977), Powers and Pershan (1977) determined with ever increasing exactness the phase diagram for dipalmitoyl phosphatidylcoline (DPPC) multilayers. The stability of the lamellar systems against mechanical disturbances made these experiments possible and several methods were applied for the determination of the phase diagrams i.e. X-ray diffraction (Janiak et al., 1976), electron diffraction (Powers, Pershan, 1977; Hui et al., 1975; Hui, 1976) deuterium NMR (Ulmius et al., 1977) differential scanning calorimetry (Janiak et al., 1976) and conoscopy (Powers and Pershan, 1977).

In our work we intended to examine the relations between the structure and function of single-BLMs (Sugár et al., 1978) therefore we focused our attention on the structure of single-bilayers. The experimental technique of studying the biologically important single-bilayer structures seems to be more complicated as compared to the multilamellar systems. In spite of the very few experimental results in literature we are now in the position to construct the phase diagram of the DPPC single-bilayer by means of some plausible considerations. Further on we discuss the experimental results for the single-bilayer structure measured at different temperatures and water contents.

Hui et al. (1975) pointed out that the only phase transition- or melting temperature of a wet unsupported DPPC single-bilayer is at 41.5 °C. This agrees with the main transition temperature 42 °C (Janiak et al., 1976) of DPPC multilayers in excess water. The spacings of the single-bilayer expand slightly from 4.2 Å

Abbreviation: BLM, bimolecular lipid membrane.

with increasing temperature until at 41.5 °C the sharp electron diffraction patterns disappear and a diffuse ring of an abruptly increased spacing of approximately 4.4 Å is obtained. This indicates that the structure of the bilayer has passed from a hexagonal crystalline packing into a liquid-crystalline structure. Hui (1976) also showed, that hydrocarbon chains of DPPC molecules are perpendicular to the surface of a wet bilayer. Thus one can conclude that an $L_\beta \rightarrow L_\alpha$ phase transition takes place at 41.5 °C, in contrast with the $P_{\beta'} \rightarrow L_\alpha$ (Janiak et al., 1976) or $L_\beta + L_{\beta'} \rightarrow L_\alpha$ (Larsson, 1977) transition of multilayers formed from DPPC.

For dried single-bilayers Hui et al. (1975) have still not reached the phase transition at a temperature as high as 62 °C. Similarly in the case of multilayers the melting temperature (T_m) is very high at low water content (T_m = 55 °C at 9 per cent water (Ulmius et al., 1977).

The amount of bound water below and above the transition temperature in single-bilayer vesicles is also comparable with the amount of bound water in multilayers. For multilayers (Janiak et al., 1976) below T_m the maximum amount of bound water is 20 per cent above T_m it is 51 per cent. For single-bilayer dimiristoyl phosphatidylcoline (DMPC) vesicles (Watts et al., 1978) below T_m the maximum hydration is 9 ± 6 per cent and above T_m it is 58 ± 4 per cent.

According to the ^{31}P NMR measurements the polar head-groups of both DPPC oriented multilayers (Griffin et al., 1978) and sonicated single-bilayer vesicles (Yeagle et al., 1977) are extended parallel to the bilayer surface.

Due to the above similarites one can assume similar water-lipid interaction in both types of BLMs. Thus the stable well-ordered lipid-water interface structure of the single DPPC bilayer will be formed at 20 per cent water content as in the case of the multilamellar system. As Forslind and Kjellander (1975) pointed out the ordered structure at 20 per cent water content is determined by the nature of lecithin and water and is fairly independent from the presence of other lamellae.

The formation of this stable interfacial structure hinders the further decrease of the thermostability of the crystalline phase. Consequently it can be stated that

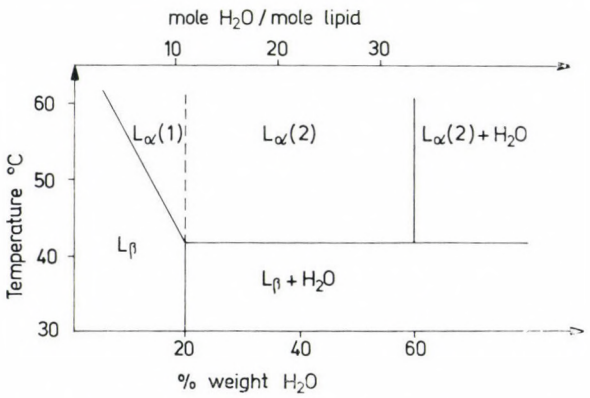

Fig. 1. The proposed phase diagram of the single-bilayer formed from DPPC

T_m gradually decreases with increasing water content and from 20 per cent H_2O it remains constantly 41.5 °C.

After these considerations the following phase diagram is proposed for DPPC single-bilayers (see Fig. 1).

In Fig. 1 a distinction is made between L_α below and above 20 per cent water content. Here we suppose that the existance of the ordered interfacial structure has to be reflected at macroscopic level too, as in the case of multilayers. For oriented multilayers Powers and Pershan (1977) pointed out a sudden change in the magnitude of the birefringence at 20 per cent water content. Gary-Bobo et al., (1971) observed a sudded change in lateral diffusion also at approximately 20 per cent water content in an egg lecithin oriented multilamellar system. According to the P^{31} NMR results (Griffin et al., 1978; Yeagle et al., 1977) the above mentioned observation cannot be explained by the changes in the head-group orientation.

Presumably single-bilayers formed from other synthetic lecithins have phase diagrams similar in their basic features.

I am grateful to Prof. I. Tarján, Drs Gy. Rontó and S. Györgyi for helpful discussions.

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Book Reviews

Stefan Bleecken: *Populationsdynamik einzelliger Mikroorganismen, Modellbildung und -anwendung*. Fortschritte der experimentellen und theoretischen Biophysik, Band 23. VEB Georg Thieme Verlag, Leipzig, 1978 (in German).

The aim of population theory is to provide a quantitative description and analysis of the causal relations between the growth of a population, the characteristics of the individuals within the population and the effects of the environment. This book deals with the behavior of the colonies of procaryote and eucariote modanes with the aid of mathematical models.

The second chapter after the introductory first one details the author's model-forming principles by presenting former papers. The most delicate problem in the construction of a model is to separate those characteristics of the constituting individuals which are essential or negligible from the point of view of the dynamics of the whole population. Stefan Bleecken's work is based on the self-reproduction of the microorganisms, so in the third chapter he briefly summarizes our present knowledge of the reproduction system of procaryotes and eucaryotes, and works out a general structural model for it. This construction is a chain of abstract processes (in concrete cases: initialisation, DNS replication, etc.), which is — in procaryotes in part, in eucaryotes wholly — cyclic. The fourth chapter introduces the definitions of the notions giving a precise description of the reproduction of cells, and through it of the dynamics of population. In the fifth chapter the author sets up his deterministic model by the mathematical formulation of the relationships. The solution of a partial system of differential equations belonging to given peripheral conditions is the "behavior-function" of the population in the surroundings determined by the conditions.

The sixth chapter contains the results of a detailed analysis of the model. We are shown that the growth is inevitably balanced, if the conditions are constant. In a theoretical way we get basic relationships between the kinetic parameters of the change in the number of cells, the velocity of the production of biomass, the time of cycle and the phases of reproduction, which harmozine with the experimental observations. After this the consequences of the sudden change of the environmental conditions (inhibition of DNS and protein synthesis, changes in temperature and in nutritive material) are treated in details. The structural difference between the system of reproduction of procaryotes and eucaryotes — which is responsible for the different reactions to the same effect — is clearly demonstrated.

Some phenomena of the dynamics of a population (e.g. multiplication starting from a single stock cell, transition from synchronic growth to asynchronic) can be studied with a stochastic model interpretable on the basis of biological variability. Chapter seven develops the previous model into a stochastic one, the eighth chapter amplifies the inferences gained in a deterministic way on the basis of the examination of the new model.

Chapter nine contains statistics which serve for characterizing the behavior of the population which is less complicated and at the same time less detailed than the previous

ones. The author shows the problems which occur at the estimations of different parameters. The relationships between the estimated values of time of cycle, time of generation, time of interdivision and phase parameters are presented on the basis of theoretical considerations.

The appendix contains not only useful tables of probability theory, but also mathematical deductions, which would break the train of thougths if inserted into the main text, but which at the same time, are indispensable for corroborating the statements.

The book can serve as a methodological guide for writing theoretical studies; the was Bleecken comes to declare the main conclusions from raising a practical problem through theoretical generalization, to forming, controlling and analysing the models is very illuminating. The mathematical means which are applied here are serious and yet perspicuous — owing to the balance between the theoretical and practical aspects of the work. A great merit of the book is its suggestiveness; the figures make even the most difficult notions clear and the most complicated conclusions perspicuous. The work is carefully constructed, systematic (though some notions of theory of systems in the sixth chapter give a somewhat strange impression and exact — just like a theoretical work is expected to be.

This book is an excellent handbook for biologists dealing with the population of unicellular microorganisms for the systematization and evaluation of their observations, and it is a useful manual for the future experts of applied mathematics as well.

J. PÓSFAI

Ch. Pilgrim (ed.) *Acta Histochemica, Band 20: Neue Methoden zur Untersuchung des Zellkernes* (Verhandlungen der Gesellschaft für Histochemie auf dem XIX Symposion im Pathologischen Institut der Universität Gent (Belgien) vom 25. bis 28. September (1977). VEB Gustav Fischer Verlag, Jena, 1979. 349 pages, 80 Figures and 26 Tables. 110. — DM

The importance of the nucleus in all the vital processes of the cell makes it imperative for cyto- and histochemists to develop new techniques for the study of the structural aspects of the molecular machinery underlying genetic phenomena and the regulation of protein synthesis. The very beginnings of quantitative histochemistry are closely related to investigations performed on the nucleus. The 19th symposium of the German Histochemical Society is an impressive cumulation of new techniques, as demonstrated by posters and in workshops. Automation and data processing in cytophotometry, with special regard to the quantitation of chromosomal apparatus in the nuclear structure and function, was one of the main topics of the symposion. On the other hand, new techniques are described for the electron microscopy of nucleic acids, especially the use of electron microscopic radioautography. From the point of practical medicine, very important are studies for specific demonstration of individual chromosomal portions. Finally, new techniques were developed for isolation of cells and subcellular particles. In all these topics, the contribution of histochemistry appears to be unique in the sense that it reveals a close correlation between structure and function.

The most important papers of the volume, comprising more than 40 contributions are as follows.

T. Caspersson, of the Karolinska Institute, described the development of quantitative cytochemical techniques for investigation of cell nuclei. Such studies were commenced in 1936, using a photographic microspectrophotometric method for studying mitotic chromosomes and also individual bands of polytene chromosomes. The recent trend in this field is illustrated by a special rapid recording fluorometer in which close to 30,000 chromosome profiles are recorded.

The next two papers are those awarded by the Robert-Feulgen-Prize in 1976 and 1977. One (ba W. D. Kuhlmann of Heidelberg) describes a peroxydase technique for labelling antigens and antibodies for immune enzymatic localization. In the other prize-winning paper (by P. Kjellstrand, Lund) the molecular mechanism of the Feulgen acid hydrolysis is analyzed.

In the next part of the issue, the papers presented in the four workshops are published, illustrated with excellent photomicrographs and/or diagrams. Workshop 1 (13 papers) was devoted to the methodological

basis of the histochemistry of the nucleus, with special regard to cytophotometry, ultrahistochemistry, and electron microscopic radioautography. Moderators of this workshop were H. Roels and F. Roels (Gent). Workshop 2 (4 papers) dealt with the analysis of chromosomes and chromatin, moderated by Lore Zech (Stockholm) and W. Hennig (Tübingen). In Workshop 3 (4 papers) techniques for isolation of cells and nuclei were presented, moderators being P. Drochmans (Bruxelles) and W. Meier-Ruge (Basel). Finally, application of histochemical methods in the diagnosis and therapy of tumours was discussed in Workshop 4, moderated by G. Kiefer and W. Sandritter (Freiburg i.Br.). In this workshop, the main emphasis was laid upon automated chromatin analysis and on the effect of antimitotic agents.

The last part of the volume is reserved for free papers, dealing with various aspects of lightand electron microscopic histochemistry. The wide spectrum of these papers yields information on the research work performed recently by German histochemists, both in the fields of normal and pathological histology. High technical level and creative application of well-based technologies characterize most, if not all, studies.

In summary, Supplement XX of Acta histochemica is an excellent reference work for all those interested in the recent *state of art* of nuclear cytochemistry. Careful editorial work and excellent typography concur with the motto of Fischer Verlag: *Semper bonis artibus*.

B. Csillik

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1980. I. 9. — Terjedelem: 7 (A/5) ív, 34 ábra

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Contents

30. IX. 1980 HU ISSN 0001—5253 Index: 26.030

VOLUME 15
NUMBER 2
1980

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

ABBPAP 15 (2) 81—163 (1980)

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (2), pp. 81—88 (1980)

Actin-Myosin Interaction in Oriented F-actin Filament System

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(Received October 2, 1979)

The decorated actin technique combined with the spin label method was used to study the interaction of skeletal muscle actin and myosin in synthetic filament system. It follows from the orientation dependence of the EPR spectra of the rigidly attached spin label in F-action filaments that the spin-label is sensitive to the dynamic state of the filaments. The interaction of myosin heads with actin reduces the intrinsic order of the F-actin filaments. The interaction seems to be cooperative.

Introduction

Recent theories of muscle contraction have suggested that the process of force generation is achieved by the sliding of filaments of actin and myosin past one another (Huxley, 1968). Regulation of muscle contraction is assumed to be localized in the thin filaments and controlled by the presence or absence of calcium ions (Ebashi, Endo, 1968). According to the proposed mechanism for muscle contraction the key problem is the interaction of myosin and actin. Recently important advances have been made in this area with different techniques (Ando, Asai, 1979; Borovikov, Chernogriadskaia, 1979; Fraster et al., 1975; Mulhern, Eisenberg, 1978; Thomas et al., 1975a; 1975b; Yanagida, Oosawa, 1978).

It was found that the conformational changes in the actin filament induced by interaction with heavy meromyosin or subfragment-1 exhibited a non-linear character so they were interpreted as cooperative conformational changes. But in most of the studies the results indicating cooperative conformational changes depend on the method used in the experiments (Tawada, 1969; Fujime, Ishiwata, 1971; Loscalzo et al., 1975).

Muscle is highly organized structurally, therefore, it is supposed that the effect of localized conformational changes induced by protein interaction or inter-filament interaction can be propagated by long-range forces to additional segments of proteins or other proteins and the interactions may differ from the interactions experienced in diluted solvent systems. The study of conformational changes in supramolecular complexes, such as in oriented filament system, is more relevant and can contribute to the better understanding of contraction, the basic problem of muscular activity.

In this paper we describe that the binding of myosin heads to actin in the absence of regulatory proteins produces changes in the orientation dependence of EPR spectra of spin labelled aligned F-actin filaments, which are due to a co-operative effect. A preliminary account of some of these findings has been presented (Belágyi et al., 1979).

Materials and methods

Heavy meromyosin (HMM) was isolated from tryptic digest of myosin according to the method of Lowey et al. (1969) and was lyophilized until use. Actin was extracted in the monomeric state (15 min) from an acetone dried muscle powder of rabbit skeletal muscle at 0 °C with 0.2 mM ATP, 0.2 mM $CaCl_2$ and 5 mM Tris HCl buffer at 8.5 pH, in the presence of 0.5 mM dithiothreitol. The extraction procedure took 15 minutes. The crude actin was purified by repeated polymerization-depolymerization cycles. Actin was labelled in F-state in 100 mM KCl and 0.6 mM $MgCl_2$ with the maleimide spin label. The spin label was purchased from Syva (USA). One mole of label was reacted with one mole of actin at 0 °C for 30 min under continuous stirring. The unreacted labels were removed by exhaustive dialysis against the extracting solution.

The spin labelled actin was polymerized again and different amounts of HMM were added to the F-actin solution under stirring. The solution was centrifuged at 100.000 x g and the pellet was used for EPR measurements.

The EPR measurements were carried out on aligned F-actin pellet at room temperature on the surface of a flat cell using a ER 9 spectrometer (Zeiss, GDR). The degree of order in the synthetic filament system was controlled by scanning electron microscope (JEOL TEMSCAN 100 C, Japan).

Results and discussion

Spin labelling of F-actin results in an EPR spectrum which is the superposition of EPR spectra from strongly immobilized labels and from a small amount of weakly immobilized labels. The fraction for weakly immobilized labels was in the order 6–7% determined by subtraction and double integration (Fig. 1). The EPR spectrum for strongly immobilized labels arises from labels which are rigidly attached to the cysteine-373 residue in actin (Belágyi et al., 1978; Lin, 1978). This fraction is very sensitive for G → F transition of actin and depends on interaction with heavy meromyosin (Stone et al., 1970; Ignatyeva, Ruuge, 1972; Stone, 1973). Moreover, in ordered F-actin filament system the EPR spectrum exhibits a strong dependence on orientation of filament axis relative to the direction of static magnetic field (Burley et al., 1972). The orientation dependence of EPR spectra in synthetic F-actin filament system suggests that the $2p\pi$ molecular orbitals of the unpaired electrons of the nitroxide labels are oriented more nearly

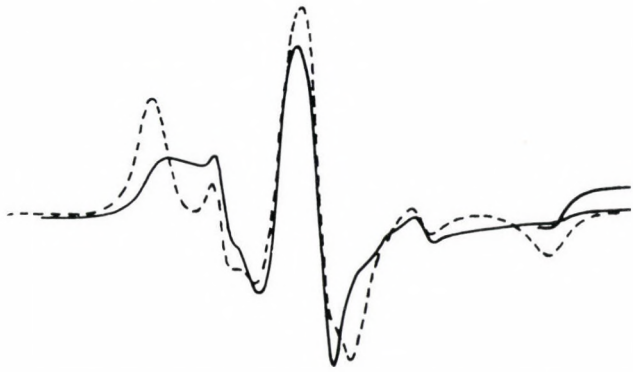

Fig. 1. Orientation dependence of the EPR spectra of aligned F-actin filaments. F-actin was labelled with the six-membered maleimide spin label. Solid line shows the spectrum where the filament axis was perpendicular to the magnetic field, whereas dotted line shows the spectrum of the same sample where the filament axis was parallel to the magnetic field

parallel than perpendicular to the long axis of the F-actin filaments and therefore the label is sensitive to the intrinsic order of filaments, which is determined by the conformational state of the entire filament.

We have introduced a simple parameter to measure the degree of order of spin labels in oriented filament system calculating the ratio

$$\alpha = \frac{H_{||}}{H_{\perp}},$$

where $H_{||} = I_{||}(A)/I_{+1}(B)$ and $H_{\perp} = I_{\perp}(A)/I_{+1}(B) \cdot I_{||}(A)$ and $I_{\perp}(A)$ are defined as the heights of the low-field peaks in the EPR spectrum for strongly immobilized labels in two different orientations, whereas $I_{+1}(B)$ is the amplitude of the low-field lines in the EPR spectra for weakly immobilized labels. The α parameter is

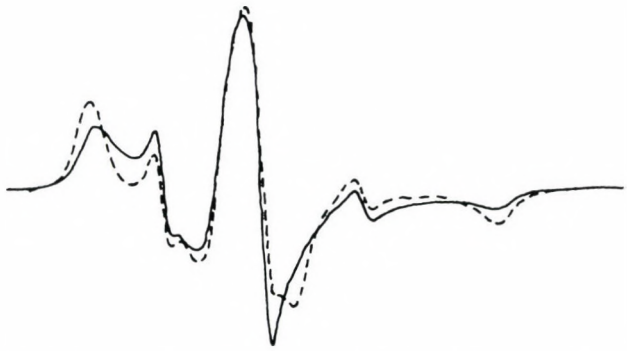

Fig. 2. Orientation dependence of the EPR spectra of the aligned F-actin-HMM system. The molar ratio of actin to HMM was 7 : 1. (------ H||k, — — — H⊥k)

not exactly correct because the height of the low-field line [I(A)] is influenced by the amplitude of the signal $I_{+1}(B)$, especially when the filament axis is oriented perpendicular to the direction of the static magnetic field.

The EPR spectrum of actin-bound maleimide spin labels exhibits a strong dependence on orientation (Fig. 1). The orientation dependence of EPR spectra is strongly influenced by the binding of myosin heads to F-actin (Fig. 2 and Fig. 3).

It can be assumed that the heavy meromyosin molecules linked to F-actin filaments cause a change in the local conformation of actin monomers. The effect of the binding of myosin heads propagates along the actin filaments by intermolecular interaction inducing changes in the intrinsic order and probably in the molecular dynamics of the F-actin, which is reflected as a decrease in the degree of order of spin labels bound to actin. Recently it was found in striated muscle that the two heads of myosin are able to interact with different actin filaments (Trinick, Offer, 1979). The cross-linking of myosin heads between adjacent F-actin filaments can promote the decrease of the intrinsic order in the F-actin filaments by interfilament interaction. The nature of response in actin filaments induced by binding of myosin heads is demonstrated in Fig. 4. It can be seen that about 0.1 moles of myosin heads to one mole of actin monomers is required to get the maximal effect, that is, the interaction induced by the binding of myosin heads is cooperative. Similar monotonic response of myosin

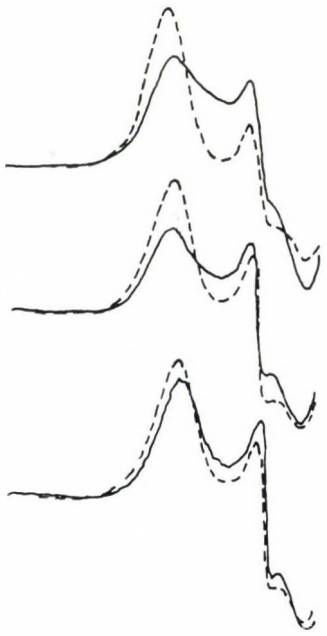

Fig. 3. Orientation dependence of the EPR spectra of aligned F-actin-HMM system at different molar ratio of actin to HMM. *a)* 10 : 1; *b)* 7 : 1; *c)* 2 : 1 mol actin to HMM. Only the two low-field lines of the spectra are shown (----H||k; — — — H⊥k)

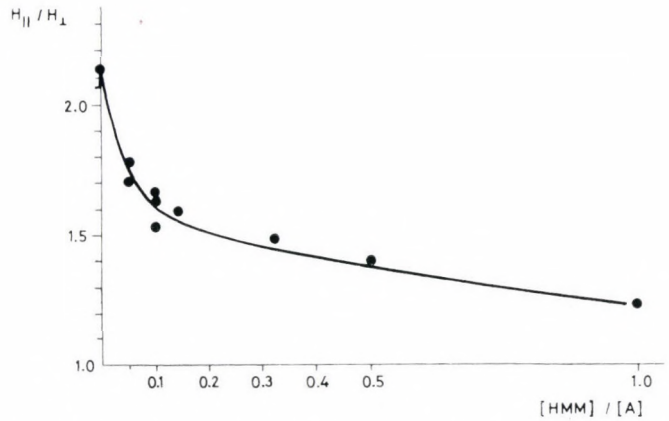

Fig. 4. The dependence of $\alpha = H_{||}/H_{\perp}$, a parameter from spectra as the molar ratio of HMM to actin. Actin is free of regulatory proteins: tropomyosin and troponin

binding to F-actin was obtained by Harvey et al. (1977) who measured the change of fluorescence lifetime of $1,N^6$-ethenoadenosine diphosphate bound to F-actin. It was also reported on spin-labelled actin using the newly developed saturation transfer EPR technique (ST EPR) that the immobilization of F-actin by myosin heads reflected similar monotonic changes, as ours, that is, the rotational dynamics of F-actin filaments is cooperatively influenced by myosin heads or subfragment-1 (Thomas, 1978; Thomas et al., 1979).

However the biphasic nature of the response was also observed in previous experiments (Loscalzo et al., 1975; Tawada, 1969). The explanations for the differences in various experiments are not clear as yet. It can be suggested that the increased interaction between myosin and actin due to the preferred orientation in multifilamental systems, such as our gel system, may differ from the interaction in highly diluted solvent systems, or the labels used in the experiments show different aspects of conformational changes in the filament system.

According to the ST EPR measurements the rotational correlation time for F-actin filament gel system falls in the sub-millisecond time range (Thomas et al., 1979). Therefore, the spectral feature can be simulated by rigid limit approximation taking into account the anisotropic distribution of spin labels with respect to the filament axis. In order to simulate these EPR spectra a new distribution function is proposed which differs from the Gaussian like distribution function suggested by Libertini et al. (1974). The distribution function has the form

$$f(\vartheta) = \frac{k_1^i \sin \vartheta}{1 + k_2^i \cos^2 \vartheta} \quad (i = 1, 2),$$

where

$$k_1^1 = \frac{k_2^1}{4\pi \operatorname{arctg} k_2^1}, \quad k_2^1 = \sqrt{p^2 - 1}$$

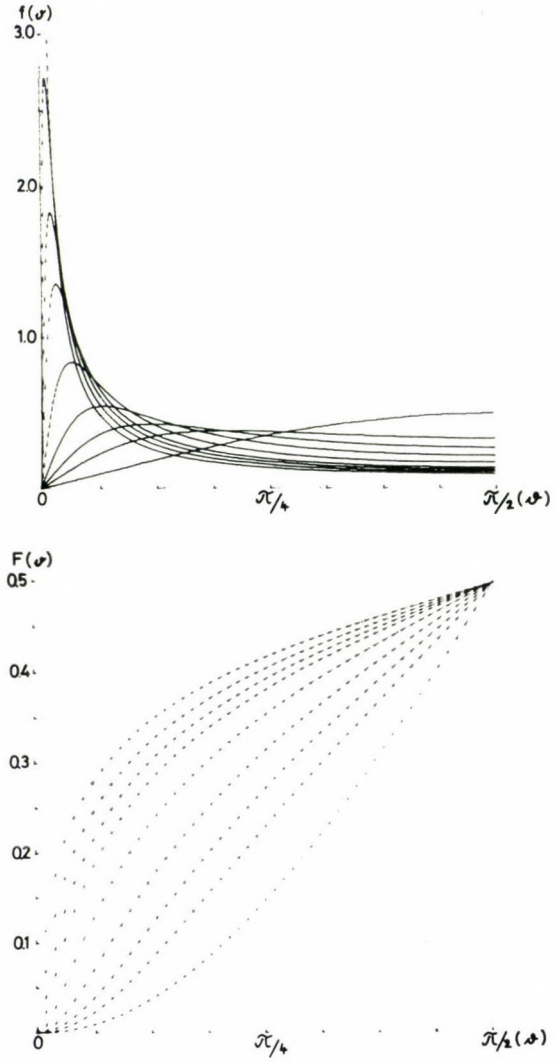

Fig. 5. *a* Density function for prolate distribution. *b* Distribution function for prolate distribution [axis ratios p (at $\vartheta = \pi/2$ from top to bottom): 1 (isotropic case), 2, 3, 5, 10, 20, 30, 50, 100]

and

$$k_1^2 = \frac{K}{2\pi \ln(1+K)/(1-K)}, \quad k_2^2 = -\frac{(p^2-1)}{p^2}, \quad K = \sqrt{-k_2^2}$$

The distribution function $f(\vartheta)$ describes the surface element of an ellipsoid of revolution (normalized to unit surface) in an arbitrary direction characterized by ϑ and gives the fraction of spin labels lying with the same principal axis within

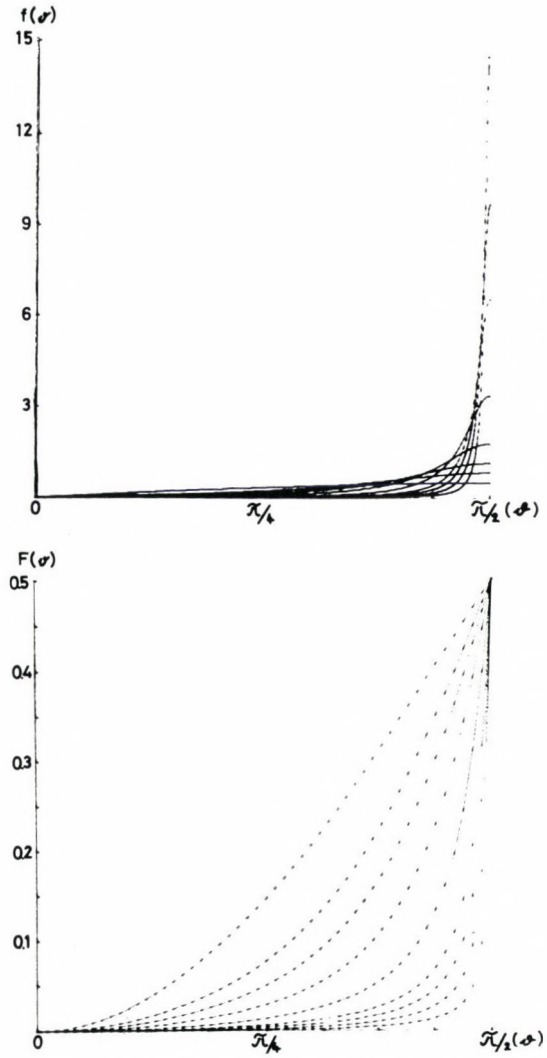

Fig. 6. *a* Density function for oblate distribution. *b* Distribution function for oblate distribution [axis ratios p (at $\vartheta = \pi/2$ from bottom to top): 1 (isotropic case), 2, 3, 5, 10, 20, 30, 50, 100]

a zone bounded by ϑ and $\vartheta + d\vartheta$. The parameter p is the axis ratio of the ellipsoid of revolution. The distribution functions for the case of oblate and prolate distributions are given in Fig. 5 and 6. Simulation of spectra for the F-actin filament system will be published elsewhere.

The binding of myosin heads to actin increases the rotational correlation time of the actin-bound spin labels. On the other hand our observations indicate

an increase of disordered domains in the filament system by interaction with HMM. Therefore, it does not seem constrained to suggest a relation between rotational dynamics of F-actin filaments and the amount of ordered domains of the multifilamental system.

In summary it can be stated that the above data in literature coupled with our observations, provide evidence that the conformational changes induced in actin on interaction with myosin heads is not restricted to one actin monomer, but the effect extends to neighbouring actin monomers too. This cooperative effect and the behavior of protein dynamics of F-actin filaments may be crucial in the muscle contraction process.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (2), pp. 89—94 (1980)

Bound Potassium in Muscle II

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(Received September 25, 1979)

Experiments were performed to decide between the alternatives *a)* the ionized K^+ is in a dissolved state in the muscle water, or *b)* a part of the muscle potassium is in a "bound" state. Sartorius muscles of *Rana esculenta* were put into glicerol for about one hour at 0—2 °C. Most of muscle water came out, but most of muscle potassium remained in the muscles. In contrast to this: from muscle in heat rigor more potassium was released due to glicerol treating than from the intact ones. 1. Supposition *a)* is experimentally refuted. 2. Supposition *b)* corresponds to the experimental results.

Introduction

Our previous paper on the state of potassium in muscle is continued in this investigation (Hummel, 1978). A model of living cell: the inorganic substances are dissolved in the water in the appropriate portion, and the biological macromolecules are also dissolved in it, and the whole is closed into a sac (e.g. Hill, 1930). An other model of living cell: the organic molecules, water, and inorganic atoms of cell are in so-called biocomplexes (e.g. Loeb, 1906).

The first conception supposes that muscle potassium exists in a solved form in the water, so if this water is extracted the potassium comes out with it in contrast to the second conception. This was the starting point in this investigation. Sartorius muscles were put into glicerol for about one hour to see how much water and potassium migrated out of the muscle into the glicerol.

Materials and methods

The experiments were carried out with sartorius muscles of *Rana esculenta*. A pair of muscles of one frog was dissected and one of them was mounted in glicerol and the parallel one was left in its normal state to serve as control. Either glicerol of 99 per cent or that of 87 per cent was used, which were precooled to 2 °C (pH 6.0 to 7.0). The bathing apparatus consisted of a thermos with melting broken ice, in which glass tubes were inserted with an aluminium mounting frame in each.

Both ends of a muscle to be bathed were tied to the mount by thread loops. The mounting frame with the muscle at its resting length was put into the glass

tube, and stayed in the ice until four muscles (in four tubes) were prepared in the same way. After pouring the precooled glicerol on the muscles the tubes were closed by rubber plugs and put back into the melting ice for about one hour at a temperature between 0–2 °C.

The other four control muscles in their intact state were weighed in a platinum jar; the glicerinated ones got from the glicerol were blotted by paper wadding and weighed in another platinum jar.

Both the glicerinated and the control muscles were dried simultaneously at 120 °C for ~12 hours, and incinerated at 450 °C for 12 hours. The ashes were dissolved, potassium and sodium contents were determined by flame photometer.

Results

1. The first question was: *how much water has got from the muscles into the glicerol*? The procedure of calculation will be outlined as follows:

the weight of sartorii in the first experiment 1.06 g;
their weight after a stay in glicerol 0.43 g;
their weight after drying 0.24 g;
the dry weight of the *control muscles* obtained from the other sides of the same 4 frogs 0.19 g.

Thus about 0.24 – 0.19 = 0.05 g of glicerol (supposedly without water) remained in the glicerol-treated muscles after drying. This must be taken into consideration, together with the fact that normal muscles – after a prolonged drying at 100 °C – still contain 1 to 2 per cents of water; moreover we can not exactly determine the actual boiling temperature of water in these systems of glicerol–water–muscle.

a) Let us make the – certainly unrealistic – supposition, that the quantity of 0.43 – 0.24 = 0.19 g, which is *lost* during the drying of the glicerinated muscles, is solely water. In this case about 22 per cent of the original 0.85 g water (0.19/0.85 ≈ 0.22) remained in the muscle, i.e. its 78 per cent diffused into the glicerol: that is an absolute minimum.

b) The other supposition is that only the part of the weight lost due to drying at 120 °C 0.19 – 0.05 = 0.14 g is water; in this case the quantity of water remaining in the muscles corresponds to 16 per cent 0.14/0.85 ≈ 0.16) of the original water content, i.e. its 84 per cent has left the muscles.

For a relatively exact calculation in case b) some special experiments had to be done in a glicerol-water-muscle system of exactly known composition in order to find out how much glicerol evaporated at 120 °C for ~ 12 hours. It was found that, under conditions similar to those of the experiments: about half of the glicerol is lost in the drying oven. For instance in the first experiment $G_d - G_d^c = 0.24 - 0.19 = 0.05$ g of glicerol remained in (and on) the muscles after drying (G_d = dried weight of glicerinated muscles, G_d^c = dried weight of control muscles); accoring to the result of the special experiments the same quantity of glicerol (0.05 g) must be evaporated. In general the weight of the water content remaining in the muscles after glicerol treatment can be calculated by the following formula: $(G_{g1} - G_d) - (G_d - G_d^c) = G_{g1} - 2G_d + G_d^c$ (G_{g1} = weight of muscles after glicerol).

Table 1

Intact sartorius muscles put into glicerol and their pairs in their normal state as control. Measured values of fresh weight of four sartorii (G_f); weight of the same 4 muscle after glicerol treatment (G_{gl}); dried weight (G_d); ash weight (G_a); and potassium and sodium contents of glicerinated muscles as well as the same values of control muscles and duration of glicerol exposure are listed. (In the first eight experiments glicerol of 87 per cent was used)

No.	g			mg			g			mg		min.
	glicerinated						control					time
	G_f	G_{gl}	G_d	G_a	K	Na	G_f^c	G_d^c	G_a^c	K^c	Na^c	
1	1.06	0.43	0.24	8.7	2.84	0.31	1.02	0.19	8.6	3.24	0.49	35
2	0.69	0.24	0.14	2.6	1.82	—	0.65	0.11	3.8	1.95	—	40
3	2.02	0.74	0.46	15.3	5.52	0.47	2.02	0.36	16.3	6.04	0.80	60
4	0.64	0.21	0.14	4.2	1.64	—	0.61	0.11	4.8	1.87	—	60
5	0.57	0.18	0.11	3.7	1.49	—	0.54	0.09	4.0	1.64	—	60
6	0.83	0.30	0.18	6.4	2.60	0.28	0.79	0.14	6.6	2.72	0.49	60
7	0.88	0.29	0.18	6.3	2.68	0.22	0.83	0.14	7.3	2.96	0.45	120
8	1.17	0.43	0.25	9.2	3.52	0.34	1.11	0.20	10.4	3.92	0.58	20
9	1.03	0.51	0.22	8.2	3.28	0.31	0.98	0.18	8.6	3.28	0.56	10
10	0.86	0.44	0.19	7.6	2.72	0.29	0.86	0.16	8.1	3.00	0.44	10
11	0.96	0.38	0.19	7.0	2.68	0.18	0.92	0.15	7.4	2.92	0.31	20
12	1.01	0.42	0.23	8.1	3.16	0.36	0.96	0.18	9.1	3.48	0.57	20
13	1.35	0.61	0.29	10.2	3.80	0.31	1.33	0.24	11.2	4.24	0.50	20
14	0.92	0.40	0.21	7.3	2.68	0.33	0.93	0.18	8.2	3.16	0.46	30
15	0.89	0.32	0.18	5.9	2.32	—	0.83	0.14	6.4	2.48	—	30
16	1.02	0.37	0.23	7.8	2.92	0.23	0.96	0.18	8.8	3.08	0.37	60
17	0.98	0.35	0.22	7.9	2.92	0.23	0.97	0.18	9.3	3.08	0.38	60
18	0.91	0.30	0.20	7.1	—	—	0.89	0.16	7.6	—	—	60
19	0.87	0.30	0.20	7.0	2.64	—	0.85	0.16	7.4	2.84	—	60
20	1.08	0.31	0.21	6.1	2.44	0.15	1.04	0.16	7.0	2.64	0.23	60
21	0.84	0.29	0.17	5.9	2.24	0.17	0.84	0.14	7.4	2.76	0.30	30
22	0.82	0.31	0.18	6.6	2.72	0.23	0.81	0.15	7.0	3.10	0.46	45
23	0.75	0.27	0.16	5.4	2.36	0.19	0.74	0.13	5.8	2.92	0.36	60
24	0.64	0.22	0.13	4.4	1.64	0.14	0.62	0.11	5.7	2.00	0.21	30
25	0.98	0.37	0.23	9.4	3.32	0.27	0.99	0.19	10.3	4.00	0.52	60
26	0.75	0.25	0.15	5.6	2.36	0.14	0.80	0.13	6.9	2.88	0.32	60
27	0.82	0.28	0.19	6.5	2.72	0.14	0.81	0.15	6.6	3.28	0.28	60
28	0.77	0.25	0.16	7.6	2.44	0.23	0.78	0.14	8.0	2.76	0.42	120
29	0.50	0.16	0.10	4.5	1.56	0.18	0.48	0.08	4.7	1.68	0.35	60
30	0.80	0.30	0.18	8.1	2.80	0.27	0.83	0.15	8.4	3.12	0.54	60
31	0.97	0.29	0.20	7.0	2.68	0.17	0.95	0.15	7.6	3.04	0.30	120
32	0.75	0.26	0.16	5.8	1.88	0.15	0.74	0.13	6.9	2.12	0.27	90
33	0.84	0.28	0.18	7.1	1.92	0.14	0.85	0.14	7.5	2.32	0.30	60
34	0.94	0.35	0.22	7.7	2.24	0.19	0.88	0.16	8.1	2.52	0.38	60
Mean	0.91	0.34	0.20	7.0	2.62	0.24	0.89	0.16	7.7	2.94	0.42	

2. The second question was: *how much potassium (or sodium) has got from the muscles into the glicerol*? The difference between the potassium (or sodium) content of glicerinated muscles and the potassium (or sodium) content of control sides was considered to be the potassium (or sodium) loss due to glicerol treatment. Sodium content was also determined as potassium and sodium are different in their ability to permeate across membranes (see Discussion).

Table 2

Calculated data of lost water, K, and Na from the glicerinated muscles in percentage of their initial contents

No.	Water	K	Na
1	78	12	37
2	82	7	—
3	83	9	41
4	86	12	—
5	86	9	—
6	83	4	43
7	85	10	52
8	76	10	41
9	61	0	45
10	62	9	33
11	77	8	44
12	77	9	37
13	72	10	39
14	74	15	29
15	81	7	—
16	83	5	38
17	84	5	39
18	86	—	—
19	87	7	—
20	88	8	35
21	82	19	43
22	82	12	50
23	83	19	47
24	82	18	33
25	83	17	48
26	84	18	56
27	86	17	50
28	86	12	45
29	85	7	49
30	81	10	50
31	88	12	45
32	84	11	44
33	86	17	53
34	84	11	50
Mean	81	11	43

Table 1 contains the weights of muscles at the beginning; their weights after glicerol treatment; dried weight in grams; weight af ashes in milligrams; and potassium and sodium content also in mg; as well as the same values for control muscles.

Table 2 contains the calculated data of loss of water, potassium, and sodium from the glicerinated muscles in percentage of their initial contents shown by the data of control sides. Here the data of lost water are the results of a) type calculation giving their absolute minimum in Table 2 (see Results 1.a.).

Summarizing the data of Table 2 it can be stated that ~81 per cent (according to b) type calculation: ~87 per cent) of the muscle water got out but ~11 per cent of the initial potassium content and ~43 per cent of the initial sodium content were lost from the glicerinated muscles.

Discussion

The results show that the majority or muscle water has got out, but most part of muscle potassium remained in the muscles placed in glicerol. Inferences: a) the conception, which considers muscle – formulated in any way – a watery solution included in a sack, and according to which this water acts only as a pure solvent of inorganic ions and organic molecules freely dissolved in it, is groundless according to our experiments. b) the other point of view, which is shared by the author, considers muscle (see above) as a system of complicated biocomplexes.* The above experimental results make this conception probable, but it is not yet proved, it does not answer to the question what the structure of these biocomplexes is exactly like, and the exact description of their properties is lacking.

The problem of permeability is naturally raised in connection with the above experiments. We are far from wishing to touch the terrible complexity and latitude of the question of permeability in the frame of this modest paper, but here the possibility of objections arises: 1. The slighter efflux of K was experienced because the wall of the muscle "sack" is impermeable to K^+ in contrast to water. – Now, it is generally accepted that muscle is more permeable for K^+ than for Na^+, therefore, beside the K-loss, also the Na-loss was determined and the latter was significantly greater than that of potassium. 2. The Na-content that got out was in the extracellular space while the K was inside the cell. – The K-loss and Na-loss do not associate with the problem of permeability or hindered diffusion according to an other investigation: the same experiments were performed on sartorius muscles in heat rigor. One leg of a frog was kept in warm water (50–60 °C) until it became rigid (3–5 minutes), the other leg was left in its normal state to serve as control. In this case, besides 77 per cent of the muscle water loss, 40 per cent of initial K-content, and 29 per cent of initial Na-content were lost from the glicerinated sartorius muscles. Thus much more K was released by mus-

* The remained main constitutents in the glicerinated muscles: potassium, vicinal water, and macromolecules may be associated in the so-called K-biocomplexes.

cles in heat rigor than in intact muscle.** This result could not be explained by a simple membrane damage due to warm water as in this case not only more K but also more Na should have been lost besides more loss of water.

Although evidence shows (e.g. Ling, 1962; Ernst, 1963; Whipple, 1965; Troshin, 1966; Hazlewood, 1973), that muscle potassium and water are "bound", it is still generally believed that K^+ is dissolved in an electrolyte solution in the cell. Some theories have been born, pro and contra, in this field but the controversy has not yet closed: Kolata; Damadian; Ling; Hazlewood; Cope and Woodbury contended in Science (1976) and a similar debate took place in Discussion Forum of Trends in Biochemical Sciences (1977). The author does not want to involve into a theoretical discussion digressing too far from our present experiments, but for all that he would like to remark that it is strange for him at any rate, that in most membrane theories only the membrane proteins (e.g. $Na^+ - K^+$/ATP-ase) are considered to have the ability to affect the monovalent cations, from this point of view the other macromolecules are ignored.

The author intends to perform further experiments on muscle potassium, keeping in mind, that the elementary biofunctions can be cleared up by investigating the cooperativity of the water content, inorganic atoms and organic molecules in biocomplexes and organoids (Ernst, 1977).

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** There are much more K-biocomplexes in "active " state of muscle than its "worn" state.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (2), pp. 95—101 (1980)

K and Ca in Muscle Mechanics II. K-Contracture

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(Received December 10, 1979)

K-rich solution, the isotony of which corresponds to normal Ringer's solution, was used in our experiments: 4.59 g KCl + 40.5 g saccharose, 1000 ml bidistilled water. Freshly prepared living muscles of Rana esculenta (m. sartorius, m. iliofibularis, m. peroneus, m. tibialis, m. gastrocnemius, m. semimembranosus) got into K-contracture in this solution, but they relaxed within some minutes. In muscles of dead frog (which canno tbe stimulated with 110 V a.c.) the K-rich solution did not elicit contracture, i.e. on the one hand K-contracture could be observed only in living muscle and on the other hand, the muscles did not lose their excitability after the K-contracture had ceased (a.c. 2V).

Introduction

The significance of the role of potassium in the mechanic and electric activity of the muscle is well known; several experts have dealt with the joint role of K and Ca in muscular activity (Heilbrunn, 1943; Ernst, 1958, 1963; Jöbsis, 1966; Gábor, Varga-Mányi, 1968). Considering only the effect of K it has been known for a long time (Grützner, 1893) that the K-content of the external medium has a significant influence on the mechanic activity of muscle. It has been demonstrated that contracture appears in isolated muscles if they are placed in K-rich isotonic solution. Contracture is characterized by a lasting retraction of the muscle under certain circumstances, which can be brought about with the aid of different chemical substances. Such substances eliciting contracture are e.g. KCl, RbCl, acetilcholine, chinine, coffeine, etc. (Fleckenstein, 1950).

Several theories are known about the origin of contracture. Rossi (1911) attributes contracture definitely to anions; in the opinion of Kuffler (1946) membrane is depolarized under the effect of the externally applied K-rich solution (80 mM KCl/l), and this is the cause of contracture; according to Bethe (1925) retraction can be attributed not to K-ions but to the chloride ion besides potassium. Riesser (1922) claims the direct effect of potassium.

In the following works the author wishes to continue the examination of the question, which has been raised previously: what is the explanation of the role of K and Ca, and their joint effect in the mechanic activity of the muscle. The aim of the present work — as the first part of a series of experiments — is to yield further data concerning the role of K in the mechanic activity of the muscle.

Materials and methods*

In our experiments muscles of Rana esculenta were used, which were placed into a K-rich solution, isotonically corresponding to normal Ringer's solution, after isolation. The K-rich solution contained KCl and saccharose in half isotonic

Table 1

M. sartorius in K-rich isotonic solution (4.59 g KCl + 40.50 g saccharose + 1000 ml bidistilled water)

Living Muscle				Dead Muscle		
length of muscle (mm)		$\frac{\Delta l}{l}$ %	excitability after contracture (V)	length of muscle (mm)		$\frac{\Delta l}{l}$ %
isolated (l)	in contracture (k)*			isolated (l)	in contracture (k)	
36	15	58	2	37	36	3
34	12	65	2	36	34	6
34	18	47	2	40	39	2
33	16	52	2	40	40	0
42	15	64	2	40	38	5
39	16	59	2	42	40	5
39	19	51	2	39	37	5
41	11	73	2	37	36	3
40	11	73	2	40	40	0
40	10	75	2	34	34	0
37	12	68	2	46	45	2
37	12	68	2	38	38	0
42	12	71	2	35	35	0
35	15	57	2	35	35	0
38	17	55	2	37	36	3
38 ±1	14 ±1	62 ±2		38 ±1	38 ±1	2 ±1

* The distance between the ends of bent muscle

quantity each. (The composition of the K-rich solution is: 4.59 g KCl (60 mM) + 40.50 g saccharose in 1000 ml of bidistilled water). The experiments were performed with the following muscles: sartorius, iliofibularis, peroneus, tibialis.

The length of resting and isolated muscles was measured in each case, and then the length of muscles – after being placed into the solution – during the contracture ensuing under the effect of K-rich solution and after the contracture was also measured with mm correctness. Furthermore the excitability of normal

* I wish to thank Mrs. Harsányi for her valuable assistance.

muscle and then the excitability after contracture was also measured. The excitability was determined by the just appreciable contraction of the muscle, stimulated directly with 2 V a.c.

In the first phase of our experiments living muscles were used. After decapitating the frog, and having measured the resting length of the above mentioned

Table 2

M. iliofibularis in K-rich isotonic solution (4.59 g KCl + 40.50 g saccharose + 1000 ml bidistilled water)

Living Muscle				Dead Muscle		
length of muscle (mm)		$\frac{\Delta l}{l}$ %	excitability after contracture (V)	length of muscle (mm)		$\frac{\Delta l}{l}$ %
isolated (l)	in contracture (k)			isolated (l)	in contracture (k)	
28	16	42	2	26	24	8
26	15	42	2	31	27	13
33	20	39	2	28	26	7
30	17	43	2	36	34	6
30	12	60	2	28	27	4
30	13	57	2	28	25	10
29	13	55	2	29	27	7
33	12	64	2	29	27	7
31	18	42	2	29	28	3
30	19	37	2	28	28	0
28	17	39	2	24	23	4
23	13	43	2	32	30	6
26	13	50	2	31	29	7
27	14	48	2	29	26	10
32	15	53	2	34	32	6
29 ±1	15 ±1	48 ±2		30 ±1	28 ±1	7 ±1

muscles, the muscles were isolated and then placed into K-rich (60 mM) isotonic solution. As it has already been described, some seconds after placing the muscles into the solution contracture occurred in the muscles, which was over in a short time.

In the second part of our experiment the muscles of dead frogs were examined. A muscle was considered dead when it was not excitable already with 110 V a.c. The circumstances of the experiments wholly agreed with those of living muscles.

Results

The result of our experiments can be found in the following tables:

Table 1.	m. sartorius
Table 2.	m. iliofibularis
Table 3.	m. peroneus
Table 4.	m. tibialis

Tables 1 to 4 show the length-data of different muscles of Rana esculenta placed into a K-rich solution. The first column under the living and dead muscles resp. contains the normal length values of the isolated muscles. The second column shows the length of the muscle placed into the K-rich solution and shortened during the contracture lasting for some seconds ($t < 1$ min). The 3-rd column contains the relative data of muscle shortening in contracture $\left(\frac{\Delta l}{l} = \frac{l - k}{l}\right)$, expressed in per cents. At the end of each column the average values together with the

Table 3

M. peroneus in K-rich isotonic solution (4.59 g KCl + 40.50 g saccharose + 1000 ml bidistilled water)

Living Muscle				Dead Muscle		
length of muscle (mm)		$\frac{\Delta l}{l}$ %	excitability after contracture (V)	length of muscle (mm)		$\frac{\Delta l}{l}$ %
isolated (l)	in contracture (k)			isolated (l)	in contracture (k)	
28	17	39	2	27	26	4
37	15	59	2	28	27	4
31	18	41	2	27	25	7
34	18	47	2	27	24	11
36	13	63	2	31	29	6
38	19	50	2	33	30	9
39	19	51	2	26	22	15
32	14	56	2	27	26	4
32	8	75	2	26	24	7
28	10	69	2	30	28	7
32	9	65	2	31	30	3
26	12	53	2	31	30	3
31	17	45	2	33	33	0
33	17	47	2	31	29	6
34	16	52	2	35	32	9
32	12	62	2	26	25	4
33 ±1	15 ±1	55 ±3		29 ±1	28 ±1	6 ±1

Table 4

M. tibialis in K-rich isotonic solution (4.59 g KCl + 40.50 g saccharose + 1000 ml bidistilled water)

Living Muscle				Dead Muscle		
length of muscle (mm)		$\frac{\Delta l}{l}$ %	excitability after contracture (V)	length of muscle (mm)		$\frac{\Delta l}{l}$ %
isolated (l)	in contracture (k)			isolated (l)	in contracture (k)	
31	19	39	2	25	22	12
29	20	31	2	28	25	10
33	18	45	2	27	27	0
29	18	38	2	23	22	4
30	21	30	2	26	25	3
27	19	30	2	28	25	10
27	14	48	2	29	28	3
32	19	41	2	28	25	10
29	18	45	2	30	28	6
24	14	42	2	32	30	6
33	20	39	2	27	25	7
30	18	40	2	30	28	6
28	18	36	2	25	23	8
30	20	33	2	27	25	7
31	21	31	2	29	26	10
30 ±1	18 ±1	38 ±2		28 ±1	26 ±1	7 ±1

Table 5

Relative shortening of different muscles in K-rich solution

Muscle	Relative shortening in per cents $\left(\frac{\Delta l}{l}\%\right)$	
	living muscle	dead muscle
Sartorius	62 ± 2	2 ± 0
Iliofibularis	48 ± 2	7 ± 1
Peroneus	55 ± 3	6 ± 1
Tibialis	38 ± 2	7 ± 1

standard deviation of the average can be seen. The data of the table show, that living muscles contract in K-rich solution; the duration of maximal contracture is ~1 minute. The degree of muscle shortening in contraction is not equal in different kinds of muscles. The muscles are excitable after the contracture.

If dead muscles are placed into the K-rich solution – in contrast with living muscles – the length of the muscles does not change significantly, i.e. no contracture appears in dead muscles under the effect of a K-rich solution.

The relative shortening of different living and dead muscles placed into a K-rich solution is presented in per cents in Table 5 together with the standard deviation of the average.

Discussion

The problem of muscle-potassium, its being bound or unbound is debated even nowadays. Similarly, there is no common attitude concerning the effect of external potassium on muscle either. It is a fact – supported by the data of literature – that a contracture occurs in the muscle if it is placed into a K-rich medium (Grützner, 1893; Riesser, 1922; Zondek, 1922; Bethe, 1925; Kuffler, 1946; Denton, 1948; Fleckenstein, 1950; Hodgkin, 1960; Lännergren, 1967; Lorkovič, 1970; Bohr, 1974). The authors give different values for the potassium concentration eliciting contracture. Zondek (1922), Lüttgau (1963), Lännergren (1967) found in their experiments that the value of the potassium concentration bringing about contracture can be influenced by several factors, e.g. the presence of another ion: Ca^{2+}.

If the potassium concentration of the applied medium is just enough to elicit contracture, the muscle shortens within some seconds and then a relaxation occurs in some minutes. The degree of contracture – as it is shown by the values of Table 5 – is different for different muscles.

Several hypotheses are known for the origin of this large-scale and quickly ceasing shortening of muscle. Several authors (Riesser, Kuffler, Bohr, . . .) consider the depolarization of membrane under the effect of K-rich solution to be the cause of contracture. Gasser (1930) brings the change of membrane potential into connection with the mechanic state of muscle. In the opinion of Taylor (1953) contractures induced in different ways do not cause any changes in membrane potential. Riesser writes about excitation-contracture. It is sure that the excitability of muscle changes significantly if it is placed into a K-rich solution (Tigyi, 1964). Bethe writes that after an advanced condition of narcosis contracture generally appears even after the complete electric unexcitability of muscle, i.e. the appearance and degree of shortening is independent of the excitability of muscle.

The experiences of our investigations with different isolated muscles of Rana esculenta are described as follows:

1. The appearance of potassium contacture depends on the excitability of muscle; in our experiments contracture was observed only in living muscles. In dead muscles, when *muscle was not excitable even with 110 V a.c. contracture did not appear in any case.*

If hind limbs of frogs were stimulated with alternating current (with the insertion of a metronome), muscle was not excitable by 30 V, only by 110 V, and in this state they were placed into K-rich isotonic solution, a relative shortening of about 5 to 10 per cent was observed.

2. In living muscles contracture appeared in each case in K-rich solutions which contain KCl and saccharose in half-isotonic quantity each as compared to Ringer's solution.

3. Some seconds after the muscle was placed into the solution a contracture could be observed, then the shortened muscle soon relaxed.

4. *Muscles which had been subjected to contracture did not lose their excitability after relaxation*, they were excitable e.g. by 2 V a.c.

The above described results suggest that some kind of connection can exist between K-contracture and muscle-excitation (see the following article). The author wishes to continue the investigation of the problem.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (2), pp. 103—107 (1980)

Transmitters and/or Metal Atoms in Muscle Mechanics

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(Received December 10, 1979)

Acetylcholine in concentrations of 10^{-5}, 2×10^{-5}, 5×10^{-5} and 10^{-4} in normal Ringer's solution causes contracture of different muscles of Rana esculenta. The similar effect brought about by KCl solution does not necessarily mean the same basic process leading to similar changes in muscle mechanics. Experiments made on many particular questions of muscle mechanics will possibly clarify the real role of single factors (e.g. transmitters, kations, anions, acids, alkalis, ATP, heat etc.) changing the mechanical state of muscular organs.

Introduction

First of all two considerations to begin with:

1. Muscles of *Rana esculenta* contain ~800 mg water and ~3.3 mg potassium per g which *a)* corresponds to a *100 mmolar K-concentration in the resting muscle*. On the other hand *b)* as generally known, *the resting muscle placed in a solution of 30—50 mmolar K-concentration goes into contracture* (Grützner, 1893; Riesser, 1925). Consequently: the potassium content of muscle is not so uniformly distributed in the muscle as in the bathing solution.

2. Acetylcholine *a) produces contracture of the striated muscle* (Riesser, 1921) and *b)* makes *heart muscle relax* (Loewi, 1921). Temporarily omitting the gigantic literature of this subject (e.g. referring to polarization, depolarization, hyperpolarization, pumps, channels, etc.) here we wish to consider two possible explanations of the opposite effects of acetylcholine: either it is itself able to exert the different actions on different biocomplexes, or it produces or changes some other constituents (e.g. K) bringing about the different effects. This latter supposition has been discussed very amply in the literature emphasizing the importance of potassium (e.g. Zondek, 1922[1]) and decidedly negated by Loewi (1921) claiming that acetylcholine acts as a "direct" transmitter.

In this paper the questions will be discussed *a)* whether the effects of acetylcholine and potassium on striated muscles are similar *and/or* different, and *b)* that metallic ions are basically important besides organic transmitters.

Materials and methods

Different muscles of Rana esculenta were used in all experiments; they were either cut out separately and put in a certain solution or a whole Läwen-Trendelenburg frog preparation was perfused with the solution and then the muscles were cut out.

The Ringer solution (NaCl 6.6, KCl, $CaCl_2$ and $NaHCO_3$ 0.2 $^0/_{00}$ each in bidist. water) contained acetylcholine in concentrations of 10^{-5}, 2×10^{-5}, 5×10^{-5} and 10^{-4}.

The muscles used in these experiments were weighed in wet and dried state, incinerated at ~450 °C in a platinum crucible; the ashes (+1 drop acet. acid) dissolved, potassium content determined in a flamephotometer.

Results

1. In experiments made for first information four years ago, the sc. Läwen–Trendelenburg preparations of 8 frogs were perfused with Ringer's solution (containing acetylcholine in 10^{-4} concentration); the left branch of the aorta was ligated, and the left mus. gastrocnemius served as control. Omitting all particulars only the end results will follow (Table 1). The *potassium loss is small* and similar to that found in experiments made in the same way when the perfusate did not contain acetylcholine (e.g. Ernst, Scheffer, 1928).

Table 1

	Weight		
	of muscle g	of K mg	mgK/g muscle
n	10.85	36.90	3.3
p	12.18	35.15	3.2

n = normal (control), p = perfused 5–6 h, 22 °C

2. Similar experiments were made this autumn with 6 frogs; Ringer's solution contained acetylcholine in a concentration of 2×10^{-5}; besides gastrocnemius also the thin muscles: sartorius, tibialis, ileofibularis and peroneus were excised and then treated together. (Table 2). Perfusion with Ringer's solution containing

Table 2

	Weight[2]	
	of muscles g	of K mg
n	5.90	22.45
p	6.87	22.34

n = normal (control), p = perfused 4.5 h, 22 °C

Table 3

Muscle		Length of muscle mm at									Weight	
		start	5"	15"	30"	1'	15'	1 h	3 h	5 h	wet g	K mg
Sartorius	n	39	39	39	39	—	—	—	37	37		
	ac	39	7*	12*	17*	—	—	—	14*	14*		
Ileofibularis	n	35	28	28	28	28	—	22	—	21		
	ac	35	18	18	20	21	—	20	—	20		
Peroneus	n	33	35	35	35	35	33	32	31	29	n 0.99	3.24
	ac	34	16	18	20	25	28	28	29	28	ac 1.14	3.54
Tibialis	n	31	30	30	30	—	31	—	29	29		
	ac	31	21	23	23	—	23	—	21	20		
Gastrocn.	n	35	35	35	35	35	—	—	31	31	1.52	4.98
	ac	35	27	26	27	27	—	—	28	27	1.66	5.34

* The sartorius muscles bend in their middle so that they take on the form appr. of a semi-circle the ends of which come nearer and nearer to each other; the figures signal the distance of the ends, the real shortening brings the muscle approximately to half of its original length.

n = normal, ac = acetylcholine 5×10^{-5}, 24 °C

acetylcholine again did not cause a substantial change in the potassium content of the muscles.

3. In view of Riesser's experimental results we performed experiments with muscles put in Ringer's solution containing acetylcholine in different concentrations. All muscles came into contracture in the first minute; the particulars of an experiment on one frog are in Table 3.

4. These results showing acetylcholine contracture are similar to those showing K-contracture (Varga-Mányi, preceeding paper, 1980). Therefore it seemed to be proper to investigate whether acetylcholine contracture can not be brought about on dead muscle similarly to the situation in K-contracture. It turned out that really acetylcholine can not induce muscle contracture on dead muscle either (dead frog's hing leg is not excitable by a.c. of 110 V).

5. The similarity between potassium- and acetylcholine contracture of muscles raises the other question of whether potassium can influence the acetylcholine contracture and vice versa. *a)* Peroneus, tibialis and ileofibularis muscles put in Ringer's solution containing acetylcholine of 5×10^{-5} concentration suffered contracture and after a few minutes began to lengthen e.g. by half of the shortening. *b)* Parallel muscles of the same frog put in the KCl solution (see also the preceeding paper) sufferend contracture and began, after a few minutes, to lengthen

e.g. by half of the shortening. Muscles from *a)* put into the solution of *b)* or vice versa did not shorten again which raise the possibility that the effects of both substances *have a similar mechanism.*

Discussion

1 Besides the papers mentioned above some others (e.g. Howell, Duke, 1908; Hemmeter, 1914; Mitchell, Wilson 1922) might have given the impression that metallic constituents as K (and Ca) are generally looked upon as playing an important role in muscle mechanism. A well-furnished scientific basis was prepared for this conception in the works especially of Hőber (1902–1914) and Loeb (1906) emphasizing the role of metallic atoms or ions in biological processes.

Thus, it meant a radical turning point in general physiological conception when the functional role of *organic transmitters* has come into prominence and the *inorganic metallic constituents* have seemingly lost their importance. And that situation came about when Loewi, applying Hemmeter's consideration, suceeded in demonstrating "the humoral transferableness of the vagus action". Only the last decades seem to have changed the general biological conception accepting the functional importance of "bioinorganic" constituents, as it is shown by the edition of e.g. Inorganic Biochemistry in two volumes (Eichhorn, 1973) and of new periodicals as e.g. Biophysical Chemistry (1973), J. of Inorganic Biochemistry (1979, earlier Bioinorganic Chemistry), Inorganic perspectives in biology and medicine (1977).

It is perhaps of some significance that very similar situation exists in the field of the physiological muscle contraction. Namely, the *mechanism of contraction* has for centuries been in the limelight of interest since Borelli (1676, 1710), (see also e.g. Engelmann, 1878; Pauli, 1898, 1912) but Hill's and Meyerhof's results (e.g. 1922–23) concerning *the heat-production* of active muscle put any other scientific particulars of muscular activity into the shade for decades. Only later, did some "inorganic" data attract more general international attention (Troshin, 1956, 1964; Ernst, 1963; Ling, 1962; Hazlewood, 1973).

2. Only in the light of the fore-mentioned particulars can one properly take in the situation concerning the problem given in the title of this paper. Fist of all, one point of view should stand quite clear: a *certain* mechanical state can be brought about by many different causes or treatments. Accordingly, the fact that contracture can not be elicited by potassium or acetylcholine on a dead muscle or on another stimulated till unexcitability may lead to the supposition that their action would be in connection with the physiological excitation of muscle. However, such unexcitable muscles can suffer several structural, chemical etc. changes of their states in which these contractures ceased to come about.

In general, all factors: transmitters, mediators, metallic kations and even some anions may play a role in the mechanical changes of muscles (see e.g. Rossi, 1911; Edman, Schild, 1962). Furthermore, even *similar* mechanical changes can

be brought about by quite different factors: shortening and lengthening by acids ("lactic acid theory of contraction"), alkalis, ATP, heat etc.

Further experiments will perhaps shed light on single particulars of the complicated problem of muscle mechanics, much less hope lays in publications containing long texts of the author's scientific cogitations instead of facts investigated experimentally.

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[1] See the "Discussion".
[2] To Table 2 the thin muscles of the six frogs.

Proceedings of the

TENTH CONFERENCE ON BIOPHYSICS

Tihany, 1979

ABSTRACTS

Contents

Active Calcium Transport in Plasma Membranes

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In most living cells the cytoplasmic calcium concentration is very low ($10^{-6}-10^{-9}$ M) as compared to the much higher calcium concentration ($\sim 10^{-3}$ M) in the extracellular fluids or in intracellular organelles such as mitochondria. The specific calcium distribution serves as a general "trigger" for initiating various cellular functions, and the normal calcium homeostasis is preserved by the plasma membrane calcium pumps: the Na−Ca exchange and/or the ATP-dependent calcium pump. In various tissues the two pumps have different shares in maintaining the steady-state calcium values. The human red cell membrane may serve as a model for studying the characteristics and regulation of the ATP-dependent active calcium transport.

A new method for investigating red cell calcium transport is the preparation of inverted, inside-out red cell membrane vesicles (IOV). This technique provides the possibility of adjusting the parameters at the cytoplasmic membrane surface, that is at the "active centre" of the calcium pump. The method enable us to study the regulation of calcium transport by nucleotides, monovalent and divalent cations and also by intracellular proteins. It could be first demonstrated in IOVs that a soluble cytoplasmic protein ("calmodulin") accelerates calcium pumping. Recently the presence of a protein, preventing calmodulin action in IOVs, has been demonstrated.

The review attempts to summarize our present knowledge about the role and regulation of the calcium pumps in various tissues.

Correlation between the Membrane-damaging Effect and Physico-chemical Parameters of Nonionic Surfactants

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The nonyl-phenyl-ethylene-oxide polymers belonging to the nonionic surfactants increase the effect of other bioactive molecules and they too show marked biological activity. To study more profoundly the correlation between the molecular parameters and biological activity of the surfactants containing 4, 6, 9, 13,

30 ethylene-oxide groups per molecule we determined their lipophility, the k and n values of Freundlich isotherm describing their surface tension decreasing effect, their diffusion constant and bactericide action on five strains. The effect of the surfactants on the ^{42}K-efflux of DPPC (dipalmitoyl-phosphatidyl-choline) liposomes and the permeability-constants were determined too. The changes in the phase transition enthalpy and temperature were measured by DSC. Between the biological activity and physico-chemical-parameters of the surfactants linear or non-linear correlations were found depending on the bacterium strains. From our data it can be concluded that surfactants interact with the lipid components of the membrane and they increase permeability.

Effect of Specific and Non-specific Proteins on the Membrane Viscosity of Intact Animal and Human Cells

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Changes in the outer membrane viscosity of sheep red blood cells and human lymphocytes, caused by reactions with specific and non-specific proteins, were studied by measuring the fluorescence emission anisotropy of the lipid-soluble dye, 1,6 diphenyl 1,3,5 hexatriene (DPH).

Blood-serum, containing specific antibodies, was found to decrease the membrane viscosity of sheep red blood cells in case the blood-serum was added to the cells before DPH. Whereas, if the same order was followed, membrane viscosity was increased by a non-specific protein i.e. ovalbumine. In the case of reverse order no significant change was observed in the membrane viscosity of sheep red blood cells.

When, however, human lymphocytes were treated with specific antibodies before the addition of DPH, the viscosity of the cell membrane was practically identical with that of the control. In the case of reverse order membrane viscosity was found to increase.

The present studies have proved that both different specific and non-specific proteins have an influence on the viscosity of the outer cell membrane. However, the tacit assumption of similar studies, that the structure of the cell membrane is not altered significantly by the incorporation of DPH, has been proved to be erroneous.

Data on the Mechanism of Primycin-Membrane Interaction

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The effects of primycin were investigated on the alkali cation transport of human erythrocytes and on the electric conduction of bimolecular lipid membranes (BLM). In the concentration range of $3 \cdot 10^{-6} - 10^{-5}$ M primycin increased the permeability of erythrocytes to alkali cations in the sequences $Cs^+ > Rb^+ \sim \sim K^+ \gg Na^+$, while the conductance of the negatively charged phosphatidylserine BLM increased by 2–3 orders of magnitude and a selectivity order $Na^+ > > K^+ > Rb^+ > Cs^+$ was found. It can be concluded from the results of our experiments that primycin interacts with the lipid phase of the membrane. The first step of this interaction is an adsorption process, then the molecule penetrates into the region of hydrocarbon chains of lipids. Using negatively charged substances (antibiotic moenomycin, SDS) and primycin simultaneously we demonstrated a charge-charge interaction between primycin and the membrane.

The Photosynthetic Properties of Membrane-modified Chlorella pyrenoidosa

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The relation of the structure of chloroplast membrane to its primary photosynthetic function was studied by the modification of chloroplast membrane using treatment with cerulenin antibiotic and pyridazinone herbicides. After these treatments the algal cells lost their normal organization: plasmolysis and fragmentation of the pyrenoid grains were observed and the chloroplast membranes became wary. The cerulenin treatment caused a strong (~30 per cent) decrease in the fatty acid content of cells while the carotenoid synthesis was enhanced. The amount of chlorophylls remained unchanged.

In spite of the structural alterations rather small changes were found in parameters related to the photosynthetic activities: the O_2-evolving activity, were the delayed luminescence and thermoluminescence not influenced practically. In cerulenin-treated cells a prominent DCMU-resistancy was observed in the absence of any antificial electron acceptor.

From these observations we concluded, that the primary photosynthetic processes can tolerate fairly strong structural changes without much disturbance in their fundamental functions. However, the normal membrane structure plays a very important role in the case of DCMU sensitivity of algae treated with cerulenin.

Examination of Membrane Parameters of Rat Heart Muscle Cells in Tissue Culture

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Experiments were carried out to determine the membrane parameters and some characteristics of action potential generation in the cells of organ and tissue cultura made from embryonic heart. The intracellular electrical potentials were recorded with conventional glass microelectrode then were digitalized for further analysis and processed with 9820 A Hewlett Packard calculator.

The first derivative of the action potential then the phase plane trajectories were computed and plotted from which the total action current was calculated. The action currents obtained were in good agreement with those expected on the basis of H–H model. The measurements were carried out in 25–40°C temperature range.

The Membrane Effect of Amines, Peptide-like Transmitters and their Relation to the Second Messenger System on the Heart of Locusta migratoria migratorioides R.F.

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Recently it has became evident that peptides can function both as hormones and as neurotransmitters as well. Tetrapeptide "proctolin" is identified in insects as a neurotransmitter but its membrane effect and its relation to aminergic mediation or second messengers are poorly understood. This relationship was studied on the isolated heart of Locusta with conventional glass microelectrodes.

Proctolin at 1.5×10^{-9} M threshold concentration caused an increase in the frequency of action potentials recorded from myocardial cells. The shape of the

action potentials was also influenced. At higher concentrations proctolin blocked the potential generation.

The effect of proctolin was not modulated by dopamine (DA) noradrenaline (NA) or octopamine (OP), but was potentiated by 5-hydroxytryptamine (5HT). This potentiation proved to be long-lasting. The membrane effect of proctolin is connected to the liberation of Ca-ions. Since 5HT is known also as a Ca-liberator it can be suggested that the potentiation was the result of liberation of Ca^{2+} from intracellular pools.

Proctolin like aminergic neurotransmitters can activate adenylate cyclase. At the same time it failed to change the activity of guanylate cyclase. 5HT caused a similar alteration in cyclase activity as proctolin did. The other three amines (OP, NA, DA) were more effective in activation of cyclases.

The effects of monoamines and proctolin on the heart membrane of insects are very similar but their mechanisms of action differ significantly.

Effect of β-Radiation on the K and Na Transport of Heart Muscle

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We have investigated the effect of β-radiation of different doses on Na and K transport and content of heart muscle. We irradiated the spontaneous and isolated frog hearts in Ringer solution containing Na^{24} and K^{42} radioactive isotopes by Sr^{90} radiation source.

As control the active hearts were treated in the solution of the same content without irradiation. Investigating the effect of 3000 rad and 6000 rad we have come to the following conclusions:

a) these doses of β-radiation do not influence the K transport and K content of heart muscle,

b) during the β-radiation of 3000 rad the Na content of heart muscle does not change,

c) 6000 rad dose increases the Na transport of heart muscle significantly,

d) under the same conditions, the Na transport of cross-striated muscle decreased under the effect of 3000 rad significantly.

Charge Movement and Mechanical Activation in Voltage Clamped Skeletal Muscle Fibers

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The relation between mechanical activation and nonlinear membrane capacity (charge movement) was studied on skeletal muscle of the frog (Rana esculenta). The experiments were done on voltage clamped cut muscle fibers (L. Kovács, M. F. Schneider; J. Physiol. *277*, 483, 1978). The currents accompanying the voltage steps were digitized and signal averaged. The final analysis was performed on R 30 computer. The linear capacity of the muscle membrane was determined from the integrals of the capacitive transients of hyperpolarizing pulses. The capacitive transients connected with depolarizing pulses represented more charge than predicted on the basis of the linear fiber capacitance. The difference between the expected and measured amounts of charge resulted from the nonlinear capacity of the muscle membrane and reflects the rearrangement of intramembrane permanent dipoles (charge movement). The time required for the mechanical movement to appear at depolarizing pulses of different sizes was measured (strength-duration curves) and the quantity of charge moved during these times was calculated (threshold charge; P. Horowicz, M. F. Schneider; Biophys. J. *25*, 201a, 1979). The amount of threshold charge decreased in the presence of 0.5 mM caffeine.

Application of Spin-Label Compounds in Membrane Research

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The Synthetic-chemistry Group of the Medical University of Pécs has synthetized more than five hundred spin label compounds during the past few years.

With the aid of EPR spectroscopy there are many possibilities for utilization of spin label compounds in the study of structure and function of biological membranes.

The most important methods are:

1. Measurement of hyperfine coupling constant.
2. Determination of the rotation correlation time.

3. Measurement of the direction dependent properties of anisotropy.

4. Determination of the distribution coefficient of spin labels between different phases.

5. Using the natural molecular constituents of the membrane labelled on specific sites.

6. Combination of specificity of immune methods with sensitivity of spin label measurement SMIA (Spinlabel Membrane Immuno-Assay).

Separation of Junctional Complexes from Nerve Cells

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Cerebral cortexes of rat brains were macroscopically excised and homogenized to prepare a crude mitochondrial fraction. In the following stages together with the densities of mitochondria increase of the buoyant iodoneotetrazolium treatment was applied. The synaptic plasma membrane fraction was separated from non synaptic and mitochondrial membrane contamination with density gradient centrifugation techniques. Synaptic junctional complexes were isolated from the synaptic plasma membrane fraction treated with Triton X-100 by centrifugation of the detergent insoluble residues on a sucrose density gradient. The purity of the obtained fractions and preservation of the membrane-pairs were observed by electronmicroscopy.

Orientation of Membran Fragments by Electric Field

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Purple membrane fragments (PM) from *Halobacterium halobium* were oriented by a static electric field. The fragments were washed in water of high purity and poured into cuvette of 0.1 cm thickness with Pt electrodes at a distance of 0.4 cm. The resistance of the solution was $2 \times 10^4\ \Omega$, the absorbance $A \simeq 1.8$ (at 562 nm). Voltages of $0-10$ V were applied through a resistance of 2 kΩ in the following time order: T (positive), 2T (zero), T (negative). T was generally 3 s. The orientation was measured by linear dichroism.

Analysing the data it was found that an electric field of ~ 20 V/cm is sufficient to achieve practically complete orientation; the PM-s have permanent electric

dipole moment of $(6 \pm 1) \times 10^{-23}$ Cm, the orientation of the retinal transition moment relative to the normal of the PM plane $\theta = (59 \pm 1)°$, and the PM rotational diffusion constant $D_{rot} = 0.65\ s^{-1}$. It was found that because of the electrophoretic movement of the particles a hydrodynamic velocity gradient builds up which also orients the PM-s. Light scattering experiments showed that the suspension was not homogeneous, it contained larger particles with smaller rotational diffusion constant $D_{rot} \simeq 0.15\ s^{-1}$.

It can be stated, that asymmetric planar membrane fragments of diameter 100–1000 nm can be oriented by rather low electric fields. Such systems – very near to the natural membranes – are suitable for the study of different membrane functions, and because of quasi crystalline order even for investigating membrane structure.

Light Scattering from Oriented Membrane Fragments

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Purple membrane fragments from *Halobacterium halobium* in water suspension were used for the investigations.

From the angular distribution of intensity of the scattered light, the average size and shape of the particles can be determined. Assuming disc-shape for the purple membrane fragments, the calculation showed heterogeneous size distribution of particles.

Orientation of the membrane fragments was achieved by a static electric field (using a square pulse of 3 sec) in the range of 0–20 V/cm. With the increase of the field strength, an excess intensity of scattered light compared to the random case appeared. Analysing the angular dependence of relaxation time, the same information was obtained, for the size distribution as from the analysis of angular distribution in the random case.

Polarization of Membranes Separating Aqueous Solutions of Different pH

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If we let the aqueous solutions of an acid and a base be separated by a permeable membrane, then polarize the acidic solution negatively and the basic solution positively, the membrane will contain the aqueous solution of the salt

composed of the anion of the acid and the cation of the base; the result will be a relatively high conductivity of the membrane ("forward direction"). Opposite polarization leads to the recombination of H^+ and OH^- ions in the membrane, thereby to the development of an insulator layer of pure water; consequently the conductivity of the membrane will be several orders of magnitude lower than in the previous case ("backward direction"). The voltage – current characteristics of the cell resemble those of semiconductor diodes ("electrolyte diode"). With the polarization of the membrane in the backward direction and a continuous increase of the ratio of alien cations in the acid or alien anions in the base, the conductivity will suddenly jump to a value corresponding to the forward direction at a definite concentration ratio, which can be determined theoretically.

Quantitative Differentiation of Intra- and Intermolecular Loosening in the Lipid Bilayer Structure. Correction of the Jacobs Model

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In contrast with the experimental findings the statistical mechanical membrane model of Jacobs et al. (Jacobs, R. E., Hudson, B. S. and Andersen, H. C. (1975) Proc. Natl. Acad. Sci. U. S. 72, 3993–3997) resulted in a volume decrease on the order → disorder phase transition of lipid bilayers. A simple correction of the Jacobs model is treated here by which this shortcoming could be eliminated. The correction strongly alters the relative area and relative volume curves but does not change the others, thus it maintains the acceptable results of the original model. The correction is applied also to our recent electric field-dependent membrane model [Sugár I. P. (1979) Biochem. Biophys. Acta 556, 72–85].

By this rewieved model the intramolecular loosening (i.e. surface area increase by the trans-gauche isomerisations in the hydrocarbon chains) and the intermolecular loosening (i.e. the change of the lateral packing relative to the closely packed case) could be calculated.

Relation between the Surface Charge and the Charge Transport Properties of Model Membranes

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The mechanism of charge carrier transport and its relation to the surface charges were investigated on oxidized cholesterol model membrane systems. The conductivity and electromotive force of bimolecular lipid membranes, and the surface potential of the monomolecular films were measured in the presence of different electrolytes (KCl, $K_3Fe(CN)_6$ and NaCl), and the surface titration of the monomolecular film was carried out as well. It was found that there are dissociable groups on the surface of the oxidized cholesterol films ($pK = 5.8 \pm 0.2$). From the experimental results we conclude that oxidized cholesterol bilayers have negative surface charges at slightly acidic and alkaline pH values (surface charge density is -0.011 ± 0.002 $As.m^{-2}$, e.i. about one charge per 1600 $Å^2$). These values may refer to carboxyl groups on the model membrane surfaces from oxidized cholesterol. As far as the conductance of the bilayer is concerned, ions of the added electrolytes do not play a direct role in the conductance of the oxidized cholesterol bilayers.

Bound Potassium in Muscle

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Experiments were performed to choose between the alternatives *a)* the ionized K^+ is in a dissolved state in the muscle water, or *b)* a part of the muscle potassium is in a "bound" state. Sartorius muscles of *Rana esculenta* were put into glicerol for about one hour at $0-2$ °C. Most of muscle water came out, but most of muscle potassium remained in the muscles. In contrast to this: from muscles in heat rigor more potassium was released due to glicerol treatment than from the intact ones. Supposition *a)* is experimentally refuted; supposition *b)* corresponds to the experimental results.

The Role of Potassium and Calcium in the Mechanic State of Muscle II

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We have already dealt with the role of potassium and calcium in muscle activity in a former paper (K. Gábor, F. Varga-Mányi, 1969). In the present work the effects of the two ions are examined separately from each other, i.e. Ringer's solutions were used, which contained either only KCl and saccharose, or only $CaCl_2$ and saccharose.

In the first phase of our experiments the role of potassium in the mechanic state of the muscle was examined. The following K-rich isotonic solution was used in our experiments: 4.59 g KCl + 40.50 g saccharose + 1000 ml bidistilled water. When freshly prepared muscles of frogs (m. satorius, m. ileofibularis, m. peroneus, m. tibialis) were placed into this K-rich isotonic solution, a contracture of the muscles appeared – in agreement with the data of literature –, which was over in some seconds.

The novelty in our experiments – in contradiction to the data published so far – is, that a contraction was observed only in excitable muscles. When the muscles were already mortified (could not be stimulated with an alternating current of 110 V), then the K-rich solution did not elicit any contracture in the muscles.

We intend to continue our examinations.

The Role of Ca^{++} Ions in the Redox Regulation of Heart Activity

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In the experiments the changes of frequency, the action potentials, the contractures and the tissue redox state potentials (E_0') of the isolated frog heart were investigated in acute oxidosis and/or redosis evoked by (through the cannula applied) oxidants (methyleneblue, thionine, safranine) or reductants (ascorbate, thiamine, cystein) in the absence of Ca^{++} ions.

In these experiments the spontaneous heart activity was inhibited by oxidants, but increased by reductants. When acetylcholine was applied the classical negative ino- and chronotropic action of this transmitter was inversed by oxidants, but augmented by reductants.

Partial absence of Ca^{++} ions in the Ringer solution resulted in a weak redosis, but this change – per se – did not evoke a spike genesis or rather contractions. In Ringer solutions with decreased concentrations of Ca^{++} ions the above described redox phenomenon failed to manifest itself, verifying that the presence of Ca^{++} ions is a necessary realizing factor for the manifestation of the redox regulation.

Measurement of Intracellular Calcium Concentration with a Metallochromic Indicator Dye on Skeletal Muscle Fibers

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Changes of the intracellular calcium concentration were measured in voltage clamped cut muscle fibers. The increase in calcium concentration accompanying depolarizing pulses was determined with a calcium sensitive dye (antipyrylazo III.; L. Kovács, E. Rios and M. F. Schneider; Nature *279*, 391, 1979). The dye entered the fiber through its cut end. The changes in the optical density of the fiber, which resulted from the penetration of the dye, corresponded well to the absorption spectrum of the dye. The calcium concentration was determined by measuring the changes in absorption of the fiber at 720 nm wavelength. The mechanical movement was eliminated by stretching the fibers (sarcomere length 3.6 μ). The effects of caffeine on the rate constants of intracellular calcium concentration changes were studied.

Modificatory Influence of Muscle Action Potential on Nerve Excitation

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As a part of our studies concerning the effects of the electrical activity of muscle on the excitation of the nerve in contact with the muscle, this work deals with the question: how can the action potential of muscle cause a change in the number of active nerve fibres in a nerve trunk. The results of the experiments performed on frog's sciatic-gastrocnemius and whole leg preparations showed that the muscle action potential brought about an increase in the quantity of the active

nerve fibres. This increase resulted in a significant augmentation of the electrical and mechanical activity of the muscle innervated by this nerve. We suppose that this kind of influence of the electrical activity of muscle on the nerve in the neighborhood of the muscles, could play a role in the regulatory mechanisms which take place in the excitatory processes of the muscle functions.

Charge of Skin Immunogeneicity under the Effect of Various Doses of Irradiation

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The efficiency of temporarily or permanently substituted tissues of the organism has been influenced unfavourably by the immunogeneicity induced by the transplanted tissue graft, in the clinical practice. It has been known from our own and others' investigations that the immunogeneicity of biological tissue grafts changed after ionizing radiation used for sterility. The present experiments were aimed at finding some relation among various doses (^{60}Co-γ) of ionizing radiation, dose-rates and the change of immunogeneicity of the rather immunogenic skin tissue.

For the determination of the immunogeneicity ration, we defined the weight of the regional lymph node and spleen of the animals in transplantation, as well as the blast transformation of lymph node cells.

It was stated that the immunogeneicity of the skin significantly decreased after 10 Krad dose of irradiation. This phenomenon was parallel to the elevation of the irradiation dose.

Preliminary experiments show that in case of similar doses this efficiency does not increase with the elevation of the dose-rate.

The Influence of DNA-Protein Interaction on the UV Sensitivity of T7 Bacteriophage DNA

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The transmission of the atmosphere for the short wavelength UV-light increases resulting from air pollution. Consequently it is interesting to study the effect of 254 nm UV irradiation on DNA and nucleoproteins respectively. Our

earlier investigations indicated that the structure and consequently the heat absorption of intraphage T7-DNA and isolated T7-DNA are different. In the present work the influence of phage protein coat and the effect of UV irradiation on the T7-DNA were studied by UV-difference spectroscopy. The difference absorption spectrum of T7 phage with respect to T7-DNA solution showed positive peaks at 279 nm and below 220 nm and a negative peak at 248 nm. The difference peaks disappeared when the T7 phage was heated above 65 °C (in M9 buffer), where the first phase transition step was localized. These results can be explained as a structural change of DNA due to release of its protein-coat. A similarity between isolated T7-DNA and T7 bacteriophage heated to 65 °C was also observed under the effect of 254 nm UV irradiation, while the intact phage seemed significantly different in this respect too. The observed difference can be explained by production of UV induced DNA-protein cross links.

The UV Photochemistry of 6-Methyluracil

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The UV photochemistry of 6-Methyluracil thin layers was studied by UV absorption and IR internal reflection spectroscopy. The spectra of unirradiated layers showed crystalline characteristics and were sensitive to the presence of water during ageing of the samples. While the effect of water molecules was pronounced in the UV region, only a slight change in the range of C-O double bonds could be seen in the IR absorption spectra. When layers were irradiated an effective photochemical reaction took place as at 250 as at 280 nm. On the basis of UV absorption only photodimers were formed. In the case of samples aged at water vapour, the reaction could be started only after a dose-gap of 1 absorbed photon/molecule. The photochemical quantum efficiency was determined based on the registration of the UV absorption spectra, and was found to be $3.5 \cdot 10^{-2}$ dimer/absorbed photon and $5 \cdot 10^{-2}$ dimer/absorbed photon in the case of 250 nm and 280 nm respectively. Thus comparing 6-Methyluracil with Uracil, an order of magnitude slower reaction could be stated in the former case. The IR absorption spectrum of the UV photoproduct of 6-Methyluracil was determined and compared with literature spectra of the isolated possible photoproducts. No contradiction with any of the dimer-type spectra could be stated, except for the presence of two sharp lines at the symmetrical stretching vibrations of the CH_3 group in our case. The exact identification of the stereoisomer, characteristic for the photodimer of solid phase 6-Methyluracil thus could not be performed.

Residual Damage of the Murine Haemopoietic System after Low Dose Irradiation

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Damage of the haemopoietic system after low-dose irradiation can not be easily detected because of the significant compensation within the system. Therefore, two methods – measurement of the proliferation rate of the pluripotent stem cells and that of the amount of mobilizable stem cells – were simultaneously applied to detect the assumed residual damage of the haemopoiesis after 0.5 Gy acute or 0.64 Gy continuous (0.16 Gy/day) irradiation.

Postirradiation reduction of the stem cell count was normalized 2 weeks after irradiation, then stem cell level oscillated around the control value. Increased proliferation rate of stem cells has been detected for 3 to 5 months after irradiation. Regeneration of the amount of mobilizable stem cells, regarded as a special subpopulation of the haemopoietic stem cells, was significantly delayed. These sensitive indicators hint at some prolonged perturbation in the structure and proliferation control of the stem cell system.

The Effect of Gamma Irradiation on the Ribonuclease Enzymes of Plants

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Seeds of pea (Pisum sativum) and barley (Hordeum vulgare) were irradiated with 0–30 Krad doses of gamma radiation from Cobalt-60 and germinated in the dark. The buds of one-week-old etiolated seedlings were used for the experiments.

The wet weights of buds decreased in proportion to the absorbed doses.

A very large increase (4 times above control) in chromatin-bound RNase was seen after irradiation. The cytoplasmic RNase activity also increased although to a smaller extent. These cytoplasmic RNases were purified by Sephadex gelchromatography and three nuclease activities were isolated. Only one of them increased after irradiation.

Measurement of Gamma Dose Component of Neutron-Gamma Mixed Radiation

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When gamma dose measurements are required in neutron-gamma radiation field, problems of separating the dose of gamma rays from that of neutrons often arise. This claim may, however, be the question of choosing the method suitable for the task. But in those cases when a gamma dosemeter is sensitive also to neutrons, its response to gamma doses can easily get unrealistic. If such type of detector as film is used, the information about the composition of radiation field leads to incorrect conclusions in biological interpretation as the consequence of the RBE values being different for neutrons and gamma radiation.

To avoid the misevaluation of high doses, the neutron sensitivity and, as the final purpose, the inaccuracy in gamma doses, measurements have been determined for the AERE/RPS film dosimetry system used for countrywide personal monitoring in Hungary. Dosemeters were exposed to mixed radiation in the biological channel of a VVR-SM reactor. Under the given experimental conditions the additional (untrue) gamma dose from activated metal filters and material of the holder was negligibly small but the KODAK RM film proved to be considerably sensitive to neutrons. As one results of the experiments, a formula has been constructed to calculate gamma doses of mixed radiation within ± 25 per cent inaccuracy under conditions similar to the experimental ones.

Effect of "Microenvironment" on the Structure of Bacteriophage Solutions (Light-scattering Studies)

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Structural damages of phage-nucleoproteins are detected by several physical-optical methods (IR-, vis.- and UV-adsorption, fluorometry) in phage-solutions. Results of these measurements can be affected by the influence of the microenvironment on the structure and the function of phages. At several values of some microenvironmental parameters (pH, NaCl concentration, temperature) changes on the "outside" structure of phages were followed by light-scattering

measurements. These structural parameters – size, shape, agglomeration – depending on the parameters of the solution were determined in the following way.

The angle distribution of the scattered light was measured at a non-absorbed wavelength (λ_0 = 436 nm) and the perpendicular scattering was measured in the function of wavelength (230–500 nm). The size and shape parameters were fitted by the Rayleigh–Gans approximation. A new method has been developed to calculate the whole scattered deficit of the absorption spectra of phage solutions in their background correction.

Experimental and Theoretical Investigations of the Action of Physiological Conditions in Chemostat Using the System E.coli-Plasmid pBR 325

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Escherichia coli GY 2354 pBR 325 was cultivated in a chemostat in different conditions in the absence of antibiotics in order to measure the curing kinetics of the multi-copy plasmid pBR 325 coding for the resistance to chloramphenicol, oxytetracycline and ampicillin. Samples withdrawn from the chemostat were tested for colony forming ability of the cells on solid medium containing the respective antibiotics. The kinetics of the increasing proportion of antibiotic-sensitive cells within the population indicated the loss of the plasmid.

The kinetics of plasmid elimination in dependence on different physiological conditions were investigated experimentally. It was stated that the elimination took place under glucose-limitation but not under ammonia limitation conditions.

A mathematical model was developed for the description of the kinetics of the plasmid segregation. In agreement with the experimental results in the analysis of the properties of this model the parameters were estimated and the conditions were found resulting in either the maintenance or the complete loss of the plasmid. Furthermore, assertions were made about possible steady states. The experimental and theoretical investigations allowed us to estimate that the number of plasmid copies was 5–8 per cell. From both the experimental and theoretical results it was concluded that the frequency of the initiation of the plasmid replication in comparison with the initiation of the chromosomal replication depends on the cultivation conditions, especially on the growth rate.

Trace Element Content of Hair along Its Length

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Some twenty five elements have been found in human hair. Most of them are valuable tools of monitoring either the metabolism or the environmental pollution of the individual. Although there exist many suitable methods for this purpose, a lot of efforts are made to apply the method of particle induced X-ray emission (PIXE) analysis because of its advantages like no need to preparate the sample, sensitivity, fastness, and multielemental property ($Z \geq 15$). Bombarding a single hair with a few nA of proton beam of 2 mm diameter and of 2 MeV energy and detecting the emitted X-rays with a Si(Li) detector one can get quite a nice spectrum within 5–10 minutes. However, it is difficult to extract quantitative data from it: either the number of the protons hitting the hair, or the bombarded mass of the hair is unknown.

We have tried to solve this problem by looking for an internal standard in the hair. For this purpose we bombarded the single hair with deuterons of 2 MeV energy. The deuterons induced many nuclear reactions and also caused the ionisation of the elements continuiting the hair. From the products of the nuclear reactions we selected the protons of the reaction of ^{12}C (d, p) ^{13}C and those of ^{14}N (d, p) ^{15}N applying two suitable detectors. At the same time we detected also the X-rays emitted after the recombination of the ions. The deuterons are not well suited particles for PIXE analysis due to the very large nuclear background but the peaks of the most aboundant elements (S, K, Ca) could be measured. The ratio of the simultaneously measured proton numbers and those of the area of X-ray peaks to the number of protons of nitrogen reaction has been determined for a lot of hairs of different persons and also along the length of the hairs. The ratio of proton numbers and that of sulphur peak to the number of protons turned out to be constant in any case within 10 per cent. This means, that sulphur can be used as an internal standard: the area of the peak of an element related to the area of the sulphur peak of the same X-ray spectrum can be used directly to compare the quantity of the trace elements in the hair of different persons.

From lengthwise measurements on many hairs we concluded that the calcium content of hair increases exponentially in moving away from the scalp, so only the part of the hair near to the root can be used to characterize the turnover of calcium in the organism. In similar measurements we found, that also the Zn content changes along the length of the hair, so Zn cannot be used as an internal standard as it has been done up to now.

The Determination of Air-born Sr-90 and I-129 Activity in Deer

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Since 1963, the long-lived fission products of the preceding atmospherical nuclear tests are supposed to have approximated dynamic equilibrium in the biosphere. For the determination of the high-grade biological accumulation of Sr-90 and I-129, deer as the monitor game seems to exhibit special advantages being able to turn over larger quantities of these radiocontaminants from its bulky food on open field, and providing bone-like samples through the antlers periodically.

Due to the diversity of their biological concentrating mechanisms and radiation characteristics, the radiochemistry of the two isotopes is quite different. Sr had to be conveniently measured by means of its daughter element, Y-90, being separated by organic solvent extraction and measured as the indefinitely thin layer of oxalate precipitate in a G – M flow counter. In this way a sensitivity of 1.5 pCi/10 g ash could be achieved. I-129, on the other hand, was determined with the aid of activation analysis, by evaluating I-128 activity over a background that needs high resolution spectrometry. Also pre- and post-irradiation chemistry has to be improved in order to get better results.

It could be concluded that the radioactive concentration of Sr-90 in different bone tissues of the deer is in the range of 1.3 – 3.2 pCi/g bone. Teeth exhibit twice as much concentration, while the compact crust of the antlers also displays the above figure thus representing well the overall Sr-activity of the skeleton. Further confirmation of why the spongious substance and even the compact root of the antlers show significantly less specific activity (below 7 pCi/gCa) than other bone tissues is still to be solved.

Study of Sr-90 Content of Human Teeth

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In connection with the establishment of Paks Nuclear Power Station, we have dealt with the measurement of the present radiation burden of the Hungarian population, as well as with the determination of "zero"-level of environmental

radioactivity. We wish to study the development of environmental Sr-90 contamination and Sr-90 concentration in human bone tissue caused by nuclear weapon tests by measuring the Sr-90 content of extracted permanent and deciduous teeth.

Teeth collected from various clinics and dentistries of Budapest, were selected according to their relatively most intensive period of development. When planning this series of experiments we started from the idea that the environmental Sr-90 activity is best reflected when the mass and parallel to this, the calcium content of each tooth has increased to the relatively highest extent. As the development of teeth practically stops after teething, they can be considered as some biological accumulator in respect of Sr-90 concentration. The concentration of Sr-90 activity was determined by way of measuring Y-90 activity by low background TESLA counter after radiochemical recovery of ashed teeth.

Having analysed the data obtained so far we may state that the annual formation of the Sr-90 content of human teeth readily follows the change of environmental Sr-90 activity. No Sr-90 could be detected in teeth cut before the nuclear weapon tests. Finally, the Sr-90 content of the bone tissue made us conclude the dose burden of the bone marrow.

Exposure of the Population to Environmental Radiation Sources

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Man has always been exposed to ionizing radiation from various natural sources. A distinctive characteristic of this natural irradiation is that it involves the entire population and that it has been experienced at a relatively constant rate over a long period of time. For this reason, exposure of the population to natural environmental sources is frequently used as a standard of comparison for exposure to various man-made sources of ionizing radiation, e.g. exposure added from nuclear power generation.

The various *natural radiation sources* include external sources such as cosmic rays and radioactive substances in the ground and in building materials, and internal sources in the form of naturally occuring radioactive substances in the human body, particularly potassium-40. The average contribution of these natural sources to the radiation exposure of human population living in areas of normal radiation background is estimated to be of the order of 1 mGy.

In some situation the exposure to natural radiation sources is enhanced as a result of *technological developments* such as travelling by air, use of some building materials, increased radon level in poorly ventilated houses, use of phosphate fertilizers, release of natural radionuclides from coal-fired conventional power plants, etc.

The most significant contribution to the exposure of the population from man-made sources stems from the radioactive fallout of atmospheric *nuclear weapons tests*. Extimates of the total global dose-commitment from all nuclear explosions performed before 1976 range from about 1.5 to 3 mGy for different tissues of man in the northern temperature zone.

The total installed capacity of *nuclear power plants* in 1976 was around 80 GW(e). The various steps of nuclear energy production result in releases of small quantities of radioactive material into the environment. This man-made contamination of the environment adds to the exposure of the population an average dose of less than 1 per cent of the natural exposure.

Determination of Cosmic-ray Charged Particle Intensity in the Lower Atmosphere in Hungary

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Many publications issued in Hungary dealt with the activity concentration of natural radionuclides, however, there are no data available on dose index intensity (D_I) of cosmic ray secondaries. We wish to supplement this lack of data our own measurements. To eliminate the disturbing effect of terrestrial radiation, measurements were performed on water surface in suitable depth and extension (over the lake Balaton at Tihany Well and also in close vicinity to Siófok) in boats and on ice using high pressure argon ionization chamber type RSS-111.

With the same chamber and exoelectron dosemeter set on board a JAK-40 plane we determined the altitude-dependence of dose index intensity. Measurements were performed in air-space above the lake Balaton, around Paks and some other place of Hungary, consequently we believe that the results represent the means over the country.

Investigation into the Radiosensitivity of T-Helper Lymphocytes

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The Immune functions of tumour bearing patients exposed to therapeutic irradiation suffer more or less damage under the effect of irradiation. The humoral immune response of the organism, the antigen production change. T-helper

lymphocytes play an important role in the combation of infections and antigen production.

The determination of these cells, *in vivo* may give more reliable information from the viewpoint of radiation therapy than examination, *in vitro*. For the sake of completing this definition a method of examination *in vivo* has been developed.

T-cell rich population was cultivated in chimeras' spleen by simultaneous addition of thymus cells and sheep red blood cells. 5 days after transplantation the activity of T-helper cells was investigated according to PFC formation of the spleen cells of untreated and immunized animals. The cortisone treatment performed on day 5 after transplantation proved that T-helper cells derived from chimeras involved some increase in the number of PFC.

Experiments showed that this system was suitable for the examination of the radiosensitivity of T-helper cells, *in vivo*. The introduction of the system has proved that it is suitable for measuring the activity of T-helper cells and the activity change in helper cells on irradiation, *in vivo* may be detected, by this means.

Radiosensitivity of Protein Synthesis of Membrane-bound Ribosomes

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Experiments were aimed at revealing the effect of the presence of the endoplasmic reticulum and the function of translation modulators acting in it, on the sensitivity of ribosomes to radiation. Authors isolated microsome and ribosome from normal and regenerating rat liver and Novikoff hepatoma cells and studied the amino acid incorporation activity of the particles after various doses of γ-irradiation, *in vitro*.

The ratio of radiation-induced inactivation gave a rather varying picture both in the presence and the absence of endoplasmic reticulum: while, in case of ribosomes the logarithm of the inactivation was in proportion with the radiation dose, the same was the reverse in microsomes, nevertheless, some correlation might be observed of a similar exponential character. Regulation factors in the membrane which induced increased protein synthesis in the presence of heavy water and in microsomes originating from regenerating liver and hepatome, lost their modulating effect preferentially during irradiation.

These phenomena prove the radiosensitivity influencing effect of the endoplasmic reticulum.

The Effect of the Compounds of Dipeptide-structure on the Survival and Capacity of Irradiated Animals

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Authors report on the radiation protection effect of some compounds of so far unknown structure. The general structure of the compounds under investigation is the following:

$$\begin{array}{l} CH_3-CH-CH-R_1 \\ \quad\quad\;\; | \\ \quad\quad\;\; S \\ \quad\quad\;\; | \\ \quad\quad\;\; S \\ \quad\quad\;\; | \\ CH_3-CH-CO-R_2 \end{array}$$

where R_1 and R_2 groups contain amino acids (i.e. glycine, alanine, valyne, leucyn, phenylalanin, prolin). The efficiency of the radiation protection compounds was measured both under pre- and postirradiation conditions. It is ratherimportant that the analogues of dipeptide structure show extremely low toxicity. Their radiation protection efficiency can be well characterized by the *Grenan* protection index. Although, the radiation protection effect of these compounds does not correspond to those of well-known compounds (i.e. AET, cisteamine), their post-irradiation effect is worthy of attention. Results obtained so far show that the radiation protection of haematopoietic organs predominates to a lesser extent according to the evaluation of the efficiency of the compound under investigation; the examined dipeptide-structure compounds protect primarily the radiation injury of the Central Nervous System well observable by the tests used for the determination of the capacity (i.e. estimation of the physical capacity, orientation activity, electronystagmography, etc.). The analysis of death curves showed that these compounds prevented the radiation injury of the gastr-intestinal tract.

Sister Chromatic Exchanges (SCEs) Induced in Human Lymphocytes *in vivo* and *in vitro* by Low Doses of Radiation

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The use of the SCE technique as a cytogenetic screening method for mutagens requires a detailed knowledge of the "spontaneous" SCE frequency in normal, healthy humans. Comparisons were made between 36 control persons (CP) and 20 individuals occupationally exposed (IOE) to chronic low doses of radiation. These data indicated a mean value of 5.36 $\pm$ 0.41 SCE/cell for CP and 8.04 $\pm$ 0.64 SEC/cell for IOE, respectively. No differences were found in chromosome aberrations in either group. Therefore, it seems that the SCE determination might serve as a checking method of low dose-effect *in vivo*.

SCE results obtained from experiments with lymphocytes cultured and exposed to ^{60}Co-gamma radiation *in vitro* with doses of 0.05 – 1 Gy showed an unexpected dose-effect relationship. There was a marked raise of SCE frequency detected between 0 – 0.1 Gy, followed by a sharp decrease between 0.1 – 0.25 Gy, while doses of 0.25 – 1 Gy caused again only a slight increase. The phenomenon observed needs further investigation if we are to explore the suitability of this method as a biological indicator of low dose radiation.

Investigation of Environmental Airbone Radioactivity

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A considerable portion of internal radiation exposure to the population is caused by inhalation of radionuclides from the atmosphere. The majority of these nuclides are attached to aerosol particles. In order to evaluate the internal radiation does adsorbed in the human respiratory tract and other organs, one has to know either the concentration or the size distribution of radioaerosol in environment.

The paper reports on investigations of radioactivity concentrations related to air volume and aerosol mass. The measurements were carried out between 1976 and 1978. Effects of atmospheric nuclear explosin and seasonal variation of radioactivity were observed. To differentiate between natural and artificial components

of activity, an estimation method was introduced on basis of disintegration of gross beta activity.

Experiments for determining the particle size distribution of radioaerosol separated by Cascade Impactor have also been reviewed. According to the results obtained, 85–90 per cent of the short-lived radon and thoron daughters are carried by the finest particles deposited on the last separatory stage.

Determination of Permittable Radioactivity Released from the Nuclear Power Station into the Atmosphere

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The present work reports on anu sually accepted mathematic model for the diffusion mechanism of radioactive materials in the atmosphere. The most important parameters of the model are reviewed. Calculations are carried out for estimating the expectable air pollution in the environment with the help of this model and the environmental factors relevant to the region of Paks Nuclear Power Station. The method determining the maximum permitted release of radionuclides significant from the viewpoint of hygiene is based on the regulations of radiation protection in Hungary, experiences gained in other countries and international suggestions.

Studies on the Radioactive Pollution of the Hungarian Reach of the Danube

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In international cooperation a series of measurements were initiated for the detection of radionuclides in the river Danube. Water, sediment, seston samples were examined at five sampling points selected along the Hungarian reach of the Danube. The results were obtained with the aid of gross-alpha, gross-beta analysis, gamma-spectrometry and radiochemical single nuclide analysis. The results of the analyses of different river-samples showed that the current radioactivity level

in the Hungarian reach of the Danube is low and is constituted primarily by natural nuclides. Some slight seasonal changes could be observed. Although the activity concentrations are very low, it is advisable to perform further sampling and measurement, and data collection with respect to the utilization of the Danube water in order to establish correctly the radiation burden of the population due to the Danube.

Investigation of the Radioactive Contamination of Filamentous Algae

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In connection with the national nuclear power programme a series of measurements were initiated for the detection of radionuclides in the Danube river. Examinations started in spring 1978 at six sampling points were selected along the Hungarian reach of the Danube. Among the various species of algae living in the Danube, the localized and well-propagating filamentous green algae (Cladophora sp., Vaucheria sp.) were chosen for investigations. The radioaticity concentrations of gamma-emitting nuclides adsorbed by algae were determined with a Camberra type (Ge)Li detector connected with a multichannel analyser. In this way the measurements of the radioactivity contents of the samples could be conducted either in wet condition or following a simple physical preparation.

The contents of ^{131}J were found in the range of 1.76 ± 0.3 to 147.0 ± 2.3 pCi/g. Similar data were obtained for the fission product-type of radionuclides. The concentration values of natural radionuclides including ^{40}K, ^{212}Pb, and ^{232}Th were between 1.5 ± 0.3 and 25.1 ± 3.4 pCi/g.

The data suggest that the filamentous green algae, due to their metabolic features and accumulation abilities, might be suitable indicators of the radionuclide contamination in the aqueous ecosystem.

Modelling of the Distributions of Discharged Radioactive Materials into the River Danube from the Paks Nuclear Power Station and Determination of Radioactivity

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Radioactive materials released from the Nuclear Power Station can cause radiation burden of man in many different ways.

Radioactive pollution gets into the environment in two ways:

1. through the stack and
2. with the cooling water.

According to the technical documentation the amount of effluent radioactivity into the Danube is small. In spite of this, calculation for dilution based on the data of the technical documentation is necessary.

At the preoperational stage we have already carried out mathematical estimations concerning the distribution of radioactivity in Danube-water.

The report gives account of the mathematical model of the calculation.

The behaviour of radioactive materials in water can be described, at some chosen assumptions, by means of a turbulent-diffusion partial differential equation of first order. By substituting the values of planned data of discharge, the dilution factors at any distance from the outlet point can be calculated. The activity shows a Gaussian distribution within a perpendicular plane put on the axis of the plum. The values of discharge maximum permitted were calculated only for the most important radionuclides concerning public health by inversion of the above mentioned differential equation.

The results of calculations indicate that the concentration values at a distance of 10 Km derivated from the values of planned discharge are 4–5 orders of magnitude less than the maximal permitted values of concentration in Danube.

Application of Gamma Spectrometry for Monitoring of Radioiodine in the Environment

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Pollution of the environment could be caused by atmospherical nuclear weapon tests, the nuclear energy industry, the manufactures and users of radioisotopes. It is generally accepted that iodine-131 is of great importance with regard

to public health. Radioiodine released into the environment gets into the human organism where it causes a higher radiation burden.

Gamma spectrometry was carried out by a Ge(Li) spectrophotometer calibrated with an iodine-131 standard. A short account is given of the rapid methods for the collection and preparation of grass samples and applied in our investigation. A typical gamma spectrum of an environmental sample taken after an atmospherical nuclear weapon test is shown in which among others iodine-131 was also detectable. A comparison is made for the recommandations of different organisations concerning the minimum detectable concentration of iodine-131.

In the course of a regular iodine-131 monitoring program it was found that the rate of pollution depends on meteorological factors on the one hand and on the other hand the character of the terrian also greatly influences the actual pollution at a given place.

Detection of Cell Surface Alterations Induced by Ionizing Radiation

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Studying the effect of ionizing radiation upon plasma membranes of mammalian cells of different origin (human fibroblasts, human and mouse red blood cells, lymphocytes, thrombocytes, etc.), we pointed out that structural and functional alterations of cell surface receptors can be detected by binding technique using radiolabelled lectins. The changes in the receptor activity proved to be dose dependent. Therefore, the phenomenon might serve as a biological indicator of radiation injury.

Diminution of ^{85}Sr-retention by Adsorbents and Radioprotectors in Whole Body Irradiated Rats

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The purpose of our studies was to investigate the retention of ^{85}Sr in whole body irradiated rats (dose rate $7.74 \cdot 10^{-2}$ C/kg ^{60}Co gamma) and to search for the optimal treatment combination of radioprotectors (cystamine, S,2-aminoethylisothiouronium-Br-HBr) and adsorbents (activated $BaSO_4$, Prontobario,

Manugel LH) administrated by gastric tube to minimize the whole body burden following ^{85}Sr internal contamination.

The elimination of ^{85}Sr was determined by external radioactivity measurement using whole body counter for small laboratory animals.

Our experimental results show that the whole body irradiation causes a reduced rate of ^{85}Sr elimination, while radioprotectors could prevent this effect with a rather good efficiency. The clearance ratio of strontium might be increased considerably with radioprotectors administrated simultaneously in a combination with adsorbents. The best results were obtained when S,2-aminoethyl-isothiouronium-Br-HBr and Manugel LH were applied together in combination.

Fate of Intratracheally Injected $^{85}SrCl_2$ in Rats Treated with Adsorbents

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Rats were intratracheally injected with 4–5 μCi (148–185 kBq) carrier free $^{86}SrCl_2$ in 0.5 ml of physiologic sodium chloride solution. Prontobario (Bracco, Italy) and Manugel LH (Alginate Ind. Ltd. England) were applied perorally as adsorbents immediately before ^{85}Sr administration. Whole body, separately collected excreta (urine, faeces) and different organs were counted for radioactivity in a whole body counter constructed for laboratory animals for 45, 10 and 3 days, respectively, in order to determine the clearance, excretion and translocation rates of $^{85}SrCl_2$. Very rapid clearance rate ($T_{\frac{1}{2}} < 1$ day) was observed in the control and other two groups treated with adsorbents after the first days of ^{85}Sr injection.

In the untreated animals, the first component was followed by a slow extended one representing a $T_{\frac{1}{2}}$ value of 62.9 ± 2.9 days. The deposition of ^{85}Sr was not significantly affected by a Prontobario treatment, however, a considerably decreased deposition could be achieved with Manugel LH administrated simultaneously. The $T_{\frac{1}{2}}$ was reduced to 39.6 ± 7.7 days and at the end of the experiment more than 70 per cent of initial body burden was depleted.

In spite of the fast translocation of radionuclide from the lungs into the bones, our results indicate that the deposition and clearance kinetics of ^{85}Sr could effectively be influenced by adsorbents especially by administration of Manugel LH.

Investigation of Extracellular Potassium Accumulation Evoked by Stimulation of the Frog Spinal Cord

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Changes of extracellular potassium concentration (ΔKe) were measured with ion-selective micropipettes in the dorsal horn of the lumber spinal segments of the frog. Both isolated and in situ spinal cords were studied. ΔKe was produced be either electrical (single pulses or short trains with 3 Hz) or nociceptive (about 1 ml water of 45–60 °C) stimulation of the hindlimb skin. 0.2–1.0 mM increase in potassium concentration could be detected at 300–600 μm depth from the dorsal surface of the spinal cord. The amplitude, time course, and spatial distribution of the ΔKe response varied with the stimulus mode. Somewhat less but clear ΔKe occurred also in the contralateral dorsal horn. Our results neither exclude nor particularly support the assumption that ΔKe would be the mechanism of presynaptic inhibition in the frog spinal cord.

The Effect of Spin-labelled Procaine Analogs on the Function of the Frog Nerve

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Procaine and its analogs inhibit the formation and propagation of the action potential in nerves. However, the way in which these agents produce their inhibitory effect is not perfectly known. Different authors have given not only different but sometimes contradictory explanations, therefore an experimental researchwork was launched to find a consistent description of the phenomenon.

Spin-labelles procaine, procaineamide, and thiocaine were used in our experiments performed on frog sciatic nerves. A nitroxide free radical was bound to the hydrophobic end of the molecules. The procaine analogs labelled this way have much the same effect, as the non labelled ones: procaine and thiocaine caused a perfect blocking of the nervous conduction, but procaineamide proved to be ineffective in this respect.

The demobilization of the labelled end of the molecules was detected by ESR spectroscopy. The rotational correlation time increased when the membrane adsorbed the local anesthetic agents. Na-ascorbate reduced immediately the free

radicals of the labelled local anesthetics dissolved in Ringer solution. This reduction took much longer time when the local anesthetics were absorbed by the nerve membrane. We think that slower reduction represents deeper embedding.

Both the increase of the correlation time and the reduction time were correlated with the blocking ability of the investigated local anesthetics.

We are grateful to O. H. Hankovszky, K. Hideg and L. Lex (Central Laboratory of Chemistry of the Medical University, Pécs) for the labelled anesthetics and for the helpful discussions.

Application of the Chemical Luminescence Method in the Investigation of the Antiradical Activity of the Se-methionin

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The basic principle of the work is, that the primer processes of the damages of the living cell caused by ionizing radiation are the same as the activization of the peroxidic oxidization processes in the lipid of cellmembrane.

By this principle, using the method of chemical luminescence an examination will be carried out concerning the ability of the Se-methionin to altering the antiradical activity of the lipids and in this way the resistance of the cells under radiation.

On the Biophysical Application of Chemical Luminescence Methods

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In the investigation of the kinetics of certain biochemical processes the methods based on the chemical luminescence have become more and more widespread.

These methods are based on the examination of the chemical luminescence curve caused by the recombination of the peroxid radical of the system. In this paper a mathematical model is introduced to make the method applicable for the investigation of the biophysical phenomena of the peroxidic oxidization.

Radioluminescence of Pyridoxin and its Derivates

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We have investigated the light emission of B_6 vitamine group, namely pyridoxin-, pyridoxal- and pyridoxamin-HCl dissolved in water at a concentration of 2–40 mM under the effect of low energy beta radiation (E_{max} = 18 keV). The radiation source was tritiated water mixed with the solution.

The solutions of the above mentioned biologically important compounds with an tritium activity of 6–54 μCi/ml emitted a well measurable light quantity in the wave-length region of 350–500 nm. The proportions of the emission intensity corresponded to the data published by Coursin and Brown (1958) for UV (330–340 nm) excitation but the intensity increased more significantly than of the concentration of materials and the activity of the added tritiated water. This was found to a large extent in the case of pyridoxamin.

The B_6 vitamin group may play a role as one of emission centers in the field of beta radiation evoked scintillations of biological objects.

Molecular Changes in DPPC Lipid Vesicles Studied with Fourier Transform Infrared Spectrophotometer

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A couple of biological events can be modelled on single wall lipid vesicles. The mechanism of these biological events is always based on the structural changes of biomolecules. The molecular vibrations are very sensitive to the structural changes. One of the best methods for studying molecular vibrations is infrared spectroscopy. Up to now this method has had great disadvantages in case of biological samples due to the infraactivity of water. The spectrum of water was superimposed on all the spectra. This problem can be solved completly with Fourier transform infrared spectrophotometer.

Our lipid vesicles were prepared from dipalmitoyl phosphatidylcholin according to the method of Kramer et al. (Biochem. *16*. p. 3932–3935). For infrared measurements we applied a Nicolet 7199 system as spectrophotometer. The samples were put into a KRS-5 cuvette with 20 μm pathway. We studied the effect of phase transition on the infrared spectra of our samples. Phase transition was induced by the changing of temperature. The spectrum of water was subtracted

from the spectra of the sample in order to get the pure spectra of the vesicles. We found that the frequency of the assymmetric and symmetric CH_2 stretching shifted towards the higher frequencies. Similar effect on the PO_2 stretching modes did not appear.

Determination of RBE and OER after 250 X-ray and Fission Neutron Irradiation

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The determination of the relative biological efficiency (RBE) of the various radiation sources and the knowledge of the oxygen effect (OER) is one of the main tasks in radiobiological research.

In experiments with mice the effect of 250 kVp X-ray and fission neutron radiation was studied in A and B types of sprematogonii of the testis as an organ sensitive to radiation.

The RBE value of A-type spermatogonii was determined; 6.8 in oxygenized conditions; 9.3 in hypoxic condition of the organ. OER was 1.41 after fission neutron and 2.19 after X-ray irradiation. RBE measured in B-type spermatogonii was 2.6 under oxygenized conditions; and 3.57 in hypoxia, at the same time. OER was 1.33 after fission neutron and 1.83 after X-ray treatment.

Differential Pulse Polarographic Studies of Primycin and its Interactions with Nucleic Acids

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Differential pulse polarography was applied to investigate the polarographic behaviour of antibiotic primycin, with a Polarographic Analyzer, P.A.R. 174 A.

The antibiotic proved to be polarographically reducible, giving the most sensitive and specific method for determinations in concentrations less than 1 μmol/l.

While investigating the polarographic behaviour of the native and denatured DNA, RNA and poly (dA-T) in the presence of primycin we demonstrated interactions. The interactions were followed by measuring the reduction potential and peak height of primycin and nucleic acids. The binding sites of primycin and nucleic acids for each other proved to be measurable in this way.

Characterization of Living Cells by Fluoresceindiacetate-hydrolyses

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The esterase activity of control and acute leukaemic AKR:Lac/F_1 hybride mouse lymphocytes was measured using fluoresceindiacetate (FDA) as fluorogenic substrate. Esterase activity was found to be nearly identical in the two types of cell. The lipophylic FDA penetrated into the normal and leukaemic cells at a similar rate, and its concentration within the cell was about a hundred times higher than its extracellular concentration. Fluorescein, the intracellular accumulation of the fluorescent product of hydrolysis, pointed to significant differences between the normal and leukaemic lymphocytes. With a fluorescence activated cell sorter (FACS III) the leukaemic cells were found to contain on an average three times larger amounts of fluorescein. Normal and leukaemic lymphocytes could be identified by measuring the light scattering and the fluorescence intensity of the cells simultaneously. Our aim is to detect leukaemic lymphocytes with the help of the FACS III at an early stage of disease, and to adopt this method for the diagnosis of proliferative disorders in the human haemopoietic system.

Flow-fluorimetric Study of Dye Uptake by Living Cell Populations

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The staining properties of membrane and DNA fluorescent markers (1,6-diphenyl 1,3,5 hexatriene, DPH; Hoechst 33342 respectively) were studied with a fluorescence activated cell sorter (Becton–Dickinson FACS III) using human peripheral lymphocyte, normal and leukaemic mouse (AKR:Lac/F_1 hybride) lymphocyte suspensions, as well as cell suspensions of human embryonal fibroblast cultures.

On mixing DPH-stained and unstained cells the intracellular concentration of the water-insoluble stain became equally distributed in about half an hour. Our data were compared with findings reported by others, and the mechanism of the intercellular translocation of stain was analyzed.

Our studies carried out with the Hoechst 33342 stain suggest that the degree of stain uptake depends not only on the amount of DNA. The fluorescence inten-

sity of certain samples changes as a function of time, and the peak, corresponding to the double DNA content in the fluorescence intensity – cell number histogram, in fact indicates the increased penetrability of chromatin towards the stain.

Analyzing the contact of cells and studying dye uptake by chromatin we attempted to characterize the mitotic and functional state of cells with a rapid, quantitative fluorescence technique.

Light Scattering Study of Molecular Interactions

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The intensity of light scattered by solutions containing different types of molecules depends among others, on the average molecular weight. On this basis the time course of reaction accompanied by increasing or decreasing average molecular weight can be followed by measuring the intensity of the scattered light. As it was proved by our studies, the activity of the glycogen phosphorylase can thus be continuously followed both in the direction of glycogen synthesis and degradation as well. This method can similarly be used for the kinetic study of other polymerizing and depolymerizing enzymes (e.g. DNA-dependent RNA polymerase, glycogen synthetase, nucleases). The method is advantageous because it requires a relatively small amount of material, and gives rather precise and detailed information about the kinetics of the processes studied. Since the intensity of the scattered light can be measured both in the visible and the UV range at any wavelengths, the reactions can be studied in reaction mixtures of any composition. Due to this possibility the method of light scattering can be used more widely than the absorption method.

A Study on Tryptophan and Pyridoxal Phosphate Fluorescence and their Interaction in Phosphorylase b

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The aromatic amino acid groups (tryptophan, tyrosine) and pyridoxal phosphate, the co-enzyme which plays an active role in the catalytic function as well, are responsible for the fluorescence of the phosphorylase enzyme. Since the emission and absorption bands of the above groups are overlapping, effective energy transfer becomes possible by means of which the intramolecular motions

and changes in conformation can be studied even in native state. In the present investigations the fluorescence of the enzyme as well as the rate of energy transfer between tryptophan and pyridoxal phosphate were studied as a function of temperature, on the following samples: native enzyme in aqueous medium, partially deuterated enzyme and the enzyme in various conformational stages (stabilized by different ligands, e.g. substrate, activator, inhibitor).

The activation energy values, obtained from the temperature-dependence of tryptophan and pyridoxal phosphate fluorescence, as well as the temperature-dependence of energy transfer rate provided information about the local fluctuations of the microenvironment of fluorofors in different conformational stages of the enzyme.

The above methods appear to be suitable for the study of the correlation existing between the intramolecular motions and the catalytic function, because the enzyme can be investigated by these methods even in native state.

A Method for the Calculation of Large Organic and Biologically Important Molecules. Pseudopotential Fragment Calculations for Model Compounds

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One of the main targets of quantum biology is the application of the theoretical ideas of quantum mechanics for large molecular systems. The semi-empirical methods are useful for this purpose, although, because of their well-known shortcomings, the results obtained by their means are not totally reliable. In order to eliminate the above problems the rather laborous *ab initio* methods demanding the large-scale use of computers, have been widely applied lately in quantum biological research.

With the application of the *ab initio* pseudopotential fragment method developed in our Institute, the quantum biological calculations have been simplified. Moreover, the energy and geometrical parameters obtained for model compounds by the pseudopotential fragment method, approximate the experimental data better than those obtained by the FGSO gragment method of Christoffersen.

The present paper describes pseudopotential molecular fragments developed for use in the formation of large organic and biologically important molecules. The good energy and geometrical estimating capability of the method is reinforced by the results of calculations made for model compounds.

Utilization of Light Energy in the Photosynthetic H_2 Production of Blue-green Alga Anabaena Cylindrica

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The utilization of the biological photosynthetic reactions for production of hydrogen from water is known as biophotolysis. The heterocystous nitrogen-fixing blue-green alga *Anabaena cylindrica* is capable of evolving H_2 under non-nitrogen fixing conditions. However, the amount of hydrogen is small; the efficiency of photosynthetic light energy conversion to the chemical energy of hydrogen is 0.1 per cent. The enzyme responsible here for hydrogen evolution is the nitrogenase which is localized in the heterocyst. In our work we have studied the possibility of increasing the amount of hydrogen by nitrogen starvation. We found that starved filaments produced more heterocysts (5 → 12 per cent) parallel with increased nitrogenase activity. In this case the efficiency of photosynthetic light energy conversion to form hydrogen was 0.2 per cent.

Pulsed Polarographic Study of the Oxygen-evolving System of Photosynthesis in Chlorella

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A pulsed polarographic device was built for measuring the amount of oxygen evolved in photosynthetic systems illuminated with light flashes. The oxygen-yield sequence of Chlorella suspensions under 30 μs flashes at a reperition rate of 2 Hz revealed that the experimental oscillatory pattern of the yield vs. number of flashes can be described theoretically with the linear four-step model (Kok's model) with the exception of the height of the yield after the first flash. The deviation of theory and experiment can be explained by the dark relaxation processes in the oxidized states (S-states) of the water-splitting enzyme, since nearly half of the reaction centers can be found in S_0-states having no positive accumulated charges in dark-adapted systems. Based upon the homogenous hypothesis for the initial distribution of the transition probabilities among the S-states, the average transition probabilities are 10, 75 and 15 per cent for the non-reactivity (misses), normal reactivity and double-reactivity (double-hits) of the reaction centers, respectively.

Electric Signals Associated with the Photocycle of Bacteriorhodopsin

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Flash-induced electric signals were measured on untreated purple membrane suspension (from Halobacterium halobium, their orientation was achieved by means of an electric field). Four components of different life-times were found which depended on the orientation and one which depended on the electric field. Additonal measurements of the time constants of the changes in light absorption made possible the unambigous assignement of the components of the electric signals to the intermediates of the bacteriorhodopsin photocycle. Similar measurements in a heavy water suspension demonstrated that the complex electric signal reflects the stepwise translocation of proton or deuteron in bacteriorhodopsin.

Temperature Dependence of Vapour Pressure of Muscle Water

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Former experiments made in our institute, and our own results have shown, that the boundedness of water remaining in gradually dried muscle increases gradually. According to this fact, evaporation heat of remaining muscle water also increases in case of gradual drying.

In our experiments we investigated the temperature-dependence of the vapour pressure of muscle water, and we counted the evaporation heat according to the Clausius-Clapeyron equation.

In case of high relative watercontent (i) the evaporation heat of muscle-water did not differ significantly from that of the water. But in case of small relative watercontent (e.g. $i = 0.05$) it increased significally.

These results have provided new information about the boundedness of biological water.

Relation of Water-binding and Molecule-size of Etilenglycol Homologs

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According to our earlier investigations as well as to the newer data in the literature the dielectric parameters of water show a change of state of water in macromolecular solutions. The change of the static dielectric constant of water cannot be considered to be proved, the relaxation time of dipolorientation increases which — according to our interpretation — changes with the microviscosity of "bulk water" parallel.

We have investigated the solutions of dioxan, etilenglycol and polyetilenglycol homologs (polymerization degree $n = 12.5 \cdot 45.5 \cdot 136.4 \cdot 454.6$) with the "dielectric microviscositymeter" we made.

The Debye relation was fitted — with computer program — to the experimental data determining the optimum of τ (relaxation time), σ_0 (d.c. conductivity) and ε_0 (static dielectric constant) parameters.

We have came to the following conclusions:

1. Monomers have smaller influence on the water than polymers.

2. Between the interval of $n = 12$ and $n = 454$ the relaxation time is independent from the size of the molecule. Probably, above $n = 12$ the cooperative effect of monotone periodic groups can be investigated.

3. There is a water content of limited rotation, the relaxation characteristic of which cannot be seen in microwave range.

4. Ion mobility data support the relation between relaxation time and microviscosity.

An Attempt to Determine the Transfer-heat of Water in Plant and Animal Tissues

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House (1974) thought there were no sufficient data for or against thermal-osmosis in cells or tissues because practically no attention has been paid to this phenomenon. The value of the transfer-heat (Q^*) characterising the measure of the effect is between -230 J/mol and $+2093$ J/mol in model experiments according to the measurements up till now ($\Delta\pi/\Delta T = Q^*/(vT)$, where $v = 1.8+10^{-5}$ $J \cdot Pa^{-1} \cdot mol^{-1}$). Ernst emphasized again and again that the thermo-osmosis

may heve an important role in water transport (1936, . . ., 1975). Indeed, there hasn't been any quantitative estimation of the Q^* in biological substance up to the present, although we previously detected the effect qualitatively on plant tissues (1963) and on hen's egg yolk (1966).

Now we created a 3 – 5 K ΔT between the living potato tissue or frog muscle and the soaking solution selectively warming them by microwave radiation (2.4 GHz), and the changes of weight were measured. The changes of weight caused by osmotic apparent Q^* was calculated by means of the equality of the weight changes: $+84 \pm 17$ J/mol on potato tissues in case of endosmosis, and -167 ± 29 J/mol in case of exosmosis. Moreover, in the latter case there was a significant correlation between the ΔT and the measure of the effect. The effect was doubtful on frog muscle.

Under certain conditions we got -63 ± 8 J/mol in isoosmotic Ringer solution, but we were not able to prove a correlation.

The Effect of Self-Association of Caffeine on its Complexation with Sodium Benzoate. A PMR Study

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The concentration dependent selective changes in chemical shifts of N-methyl protons of caffeine were measured by nuclear magnetic resonance in deuterium oxide at 35 °C. On the basis of dimer model the association constants for self-association of caffeine and for its complexation with sodium benzoate were found to be (3.8 ± 0.2) M^{-1} and (0.8 ± 0.2) M^{-1}, respectively. The findings indicate that the molecular interactions of caffeine are influenced by its self-association.

Saturation Transfer EPR Measurements on Spin-labelled Glycerinated Muscle Fibres

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Saturation transfer EPR measurements were performed on glycerol-extracted muscle fibres labelled with maleimide spin label at the SH-2 sulfhydryl site of myosin.

It is concluded that the rotational correlation time for spin label in muscle in rigor state was nearly 10^4 times higher than in isolated heavy meromyosin in

solution labelled at the same thiol site. The reduced motion of the label at SH-2 site reflected not only a strong immobilization of the heavy meromyosin in the supramolecular system of glycerinated muscle in rigor state, but also an induced immobilization of the label within the region of heavy meormyosin containing the label. The EPR spectra exhibited a strong dependence on orientation which suggest a restricted anisotropic motion of the myosin fragment in muscle.

Orientation Dependence in the EPR Spectra of Glycerol-extracted Muscle Fibres

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According to the generally accepted theories about muscle contraction the force is generated by the interaction of actin and myosin. The direct interaction of actin and myosin presupposes the flexibility of large protein segments, especially in the head region of myosin. The proposed rotational motion of myosin cross-bridges can be monitored by spin labels rigidly attached to the SH-2 group of myosin head.

The glycerol-extracted muscle fibres were washed in rigor solution containing 10^{-3} M 5,5'-dithiobis(2-nitrobenzoic acid) at 0 °C for six hours to block the more reactive SH-1 groups of myosin. The SH-2 groups were labelled with maleimide spin labels in the presence of ATP.

In isometric contraction the EPR spectra of spin labelled muscle fibres exhibited a strong dependence on orientation relative to the fibre axis, which was markedly reduced under isotonic conditions.

Investigation of Human Lenses by the Spin Probe Method

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The spin probe electron spin resonance (ESR) technique was used on normal and senile cataractous human lenses in order to study the changes in metabolism during progression of the senile cataract by the reduction of spin probes incorporated into the lenses.

Human lenses were classified as follows: 1. normal lenses; 2. nuclear cataract; 3. cortical cataract; 4. cortical and nuclear cataract; 5. completely opaque lenses. The lenses were homogenized in Krebs-Ringer solution (pH 7.40) and 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl spin probe was added to the solu-

tion at a final concentration of 10^{-4} M. The rate of reduction of spin probes was measured as a decrease in the low-field line of the ESR spectrum. The experimental data were fitted by the least squares method supposing a pseudo-first-order kinetics.

It was found that the rate of reduction of spin probes depended on the concentration of reduced glutation and of the free-radical intermediates produced by oxidation and reduction. The rate of reduction different from the different state of cataracta and the two processes could be separated by adding KCN to the solution. It is concluded that the rate of reduction of spin probes may be a sensitive indicator of the change of metabolism occurring during the progression of the senile cataract.

Ultrastructure of Myofilaments from Insect Flight Muscle

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Isolated filaments were prepared from Honey-bee (Ápis mellifica) flight muscle after the M and Z lines were removed using low ionic strength solution. The thick filaments were separated from the thin filaments by zone sedimentation in a density gradient. Negatively stained and shadowed preparations were investigated in an electron microscope.

Thin filaments found in the same preparations varied greatly in length. Besides filament fragments we found many thin filaments longer than one-half of the sarcomere length. Some thin filaments exhibited an apparent double helical arrangement of actin subunits.

The thick filaments had very regular tapered ends. Connections remained generally at the level of the former Z lines between the very regular tapered ends of two different thick filaments from opposite sarcomeres. On several occasions the tapered end of a thick filament bifurcated at the level of the former Z line and joined with two different thick filaments from the adjacent sarcomers. Very rarely thick filament was observed which continued in three different thick filaments originated from the adjacent sarcomere. Considerint the arrangement of thick filament along the Z line of opposite sarcomeres, it is very probable that every thick filament is connected in vivo by Z filaments with three different thick filaments from adjacent sarcomere.

The projections of thick filament and the subfilaments on the surface of filament were clearly seen. Under the conditions given here the elongated heads of these projections were attached to the shaft of the filaments. On several occasions between the origin of the projection and the attached site of the head the rod of

the projection formed a 5—600 Å long arch-like curve. The head position was perpendicular or angled on the shaft. These phenomenona support the assumption that there is a flexible hinge region near the head of the projection, and at the origin of the projection. In addition to this the rod of the projection can also be characterized with flexibility. The head of the projection is too large for a single myosin molecule head, therefore it is very likely that two myosin molecules play part in its make-up.

Investigation of Biophysical, Biochemical and Physiological Changes in Frost Sensitive Grape Variety under Freezing Treatment

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One of the most important questions of the Hungarian grape growing is the loss of harvest caused by frost damages. In the interest of elucidating the biological basis of freezing injury, we investigated the mechanism of freezing effect multilaterally, in case of frost sensitive grape variety (Cardinal c. v.) by applying artificial cold induction several times during the period from November till March. The freezing treatment was carried out in programmable climate chamber, at two different cooling rates, at $-17\,^{\circ}C$ end temperature.

In comparison with the freshly harvested control of the field, we examined the effect of cold induction to the state of water in the xylem (by measuring microwave dielectric parameters) and the ATP-ase activity bound to the mitochondrial membrane. The changes in the survival of buds, the water content of the cane and xylem and the water retaining ability of cane were measured in the period of dormancy.

Standardization Method Used for Comparing the Relative Radionuclide Concentration of Rat Organs Changed Due to Growth

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Authors determined the whole-body and organ-growth curves of RxLE F_1 rate used for experiments and kept them under normal conditions to study the accumulation conditions of radionuclides.

The ratio of activity per gram of the organs and the whole-body (this experimental value is decisive for the biokinetical parameters of the substance labelled with the nuclide in question) shifted due to growth and as a result, a systematic error in the coincidence of organ concentration values occurred.

With the help of analytically fixed curves of growth the whole-body and the mass of organs of rats (with respect to males weighing 250 g and females of 200 g) were decided as standards. Furthermore, the relative concentration and relative retention values of the organs were calculated on the "given conditions".

The curves of growth and standardized values gave further possibilities for the determination of the relative retention and relative concentration values obtained from scanning uptake.

Change of the Heinz-body Number after Irradiation

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Literary data supposed a correlation between the number of erythrocytes carrying Heinz bodies and the absorbed dose of radiation. Experiments were aimed at studying this phenomenon to decide whether it is – as a biological indicator – suitable for the estimation of the severity of radiation injury. The correlation between the absorbed dose and the number of erythrocytes carrying Heinz-bodies was examined in CFLP mice. Mice were exposed to ^{60}Co-gamma whole-body irradiation in 20–200 Gy dose-range. Blood samples were taken from the animals after irradiation and the relative Heinz-body number was counted. The correlation between the number of Heinz-body carrying erythrocytes and the radiation doses was found satisfactory.

On the Possibility of Image Processing Via Non-adequate Stimuli (Virtual Iconography)

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We live in a three-dimensional environment, but we generally display it on a two-dimensional surface, calling the resulting pattern "image" or "picture". We rarely ask whether this image is or is not identical with the signal pattern formed on the retina, we only believe it is. However, it can be shown experimentally that a signal pattern on the retina may be perceived as an image of two totally

different meanings. It is enough to refer to the well-known ambiguous figures and to some works of art. This means that the signal pattern conveyed to the retina by electromagnetic waves is sent along the optical nerve in a form of pattern of nerve action potentials, i.e., the original signal pattern reaches no farther than the retinal receptors.

The perceived visual image is the result of a physiological processing in which the central nervous system has to decide which signals go together, which "form" an unit object, which constitute the boundaries that delineate it, and which constitute the background. Therefore, it a signal pattern of arbitrary origin could be presented directly to the visual perception region of the brain, it could be perceived as an optical image. Furthermore, this non-adequate signal pattern would be perceived in space, and not on the input field (receptor area) of the signal pattern, in a similar way as in normal vision where the object is seen in space, and it is not perceived on the retina where the light pattern is transformed into a stimulus pattern.

The scope of this presentation is to discuss briefly whether the *direct* sensation of an ultrasonic signal pattern or the *direct* visual-like perception of an ultrasonic signal pattern could be achieved if a suitable "physiological relay" were found capable to present it to the optical perception region of the brain.

Based on the results of Bach-y-Rita and co-workers, who developed a tactile stimuli visual substitution system, we demonstrate that if the signal pattern of a high resolution ultrasonic vidicon camera (Sokolov tube) or of a real time scanner is delivered to the skin under subject's control through an array of electrode stimulators, it may be possible to learn to *identify* the insonified target *subjectively* in space, i.e., to perceive the target in a way the *virtual* image of a reconstructed hologram is perceived in space.

This is why this method is called by us "virtual iconography".

Virtual iconography renders a sensation which cannot really be compared to the normal, optical "image environment". It will be somewhat similar to the sensation that one gets if the eyes are closed and one "sees" the object in one's mind. But just this is the plus what virtual iconography could give us. It has been demonstrated that compared to 70 per cent of the primary visual cortical cells responding to visual stimulation, 39 per cent responds to sound, and 46 per cent to skin stimulation in the same area, and so, the possibility arises to establish associative connection between the perceived sound image and an optical image displayed at the same time. This, however, would give us a new informations.

Book Reviews

Theoretical Approaches to Complex Systems, Lecture Notes in Biomathematics, Vol. 21., Eds. R. Heim, G. Palm, Springer-Verlag, Berlin—Heidelberg—New York, 1978.

This book is volume 21 of the Springer Series "Lecture Notes in Biomathematics". The series, edited by S. Levin, aims to report new developments in biomathematics research and teaching, and contains both monographs and reports of meetings. They are addressed to a relatively narrow circle, and generally have high professional level.

The present volume is the issue of the symposium held in Tübingen on June 11 and 12, 1977. In these Proceedings purely mathematical studies are represented along with theoretical and experimental works related to biology.

A series of papers presents mathematical tools which may be helpful in analyzing complex systems.

In the paper by H. Hahn critical systems are dealt with. Such systems may produce mono- and bistability, relaxation oscillations, switching or storage behaviour, etc. The author presents a purely algebraic procedure to determine the stability behavior of critical nonlinear systems. Although the procedure is rather laborious, and is proven only for systems in which there exists a single simple critical eigenvalue, the demonstration of such a procedure is an important achievement.

Generalization of the results is also conjectured.

G. Palm proposes goal functions, a generalization of the Liapunov function, to investigate the asymptotic behavior of dynamic systems.

R. Heim studies optimal working regime of an information channel, if transfer of different input letters is associated with different expenses and/or some distortion of the message is tolerable.

M. Dal Cin considers probabilistic self-diagnosis of multicomponent systems, in which the components may test each other. It is shown that for some testing strategies and some test outcomes the faulty units can not be identified.

Interesting examples of 3-variable dynamic systems with "chaotic" behaviour are given by O.E. Rössler and P. J. Prtoleva. These equations, demonstrating excessive sensitivity to noise, are in principle realizable by chemical reaction networks.

In another group of papers there are models for the description of different phenomena in the nervous system.

U. An Der Heiden proposes a general system of integral equations for the treatment of neural networks Several well known models (McCulloch and Pitts, Hartline—Ratliff, Cowan) appear as special cases of the system.

T. Poggio and V. Torre describe nonlinear effects of synaptic conductance changes by Volterra integral equations. They suggest synapses to be the basic computing elements in the nervous system.

From the experiments on figure-ground discrimination by the visual system of the fly, W. Reichardt concludes that fourth-order lateral interactions are needed to explain the results. Thus, critical interplay of temporal and spatial parameters characterizes the spontaneous pattern preference, not the geometric parameters alone.

V. Braitenberg explores the possibility that the presence of a relevant event is signalled by identifiable sets of neurons, and supposes, that the pyramidal cells of the cerebral cortex may from such cell assemblies.

D. Varju presents the experimental results related to the delayed response in the retinal ganglion cell of the frog, and builds a model where the ON and OFF signals are processed separately, which successfully explains the results.

The contribution of M. Conrad is conceptually different from any of the previous articles. The properties of the adaptive landscape (i.e. of the dependence of fitness on environmental and biological variables) are searched for, considering, that evolution operates on a "single genetic event at a time" basis. It is shown that gradualness (= optimizability by stepwise evolution) is itself an evolved property.

This book is not an easy reading either for a mathematician or a biologist. Its content is more heterogeneous than used to be in this series. Both extremities typically occurring under the heading "Biomathematics" appear also in this volume: purely mathematical deductions without any reference to biology, on the one hand, and huge experimental works with a tiny *ad hoc* model, on the other. (Though these contributions also may carry important new informations, see e.g. Hahn). The contributors also discuss their subject at different depths and with different kinds of reader in mind.

Nevertheless, a couple of results and concepts are worth the attention of anybody dealing with complex (biological) systems.

Cs. Fajszi

Allgemeine Mikrobiologie. By Weide, H., Aurich, H., VEB Gustav Fischer Verlag, Jena, 1979. 519 pages, 300 figures and 47 tables.

General Microbiology by Weide and Aurich is a textbook compiled mainly for non-medical and non-veterinary students. Therefore it is quite natural that some of the topics are more detailed while others are not.

Following the first *Introductory Chapter* a short chapter summarizes the basic facts about the building elements of cells. Among these elements belong the ions and organic components as amino acids, fatty acids, monosaccharides and nucleotid bases. Some approaches of the macromolecular organization are also included.

The third chapter — *Morphology* — is more detailed describing separately the bacterial cell structure, the viral organization and fungal morphology. There is also a short summary about the important structural elements (genom, membranes, cell walls, accessoric appendages, etc.). In connection with the most important structural elements, e.g. the cell well of bacteria some data are also briefly commented on concerning the biosynthesis or the agents (EDTA, muramidase, penicillin) affecting it. In case of virus morphology the process of virion assembly or reproduction is also discussed.

The most detailed data can be found in Chapter 4: *Metabolism*. After describing some basic factors like enzymes, their mechanisms, kinetics and coenzymes he gives a summary of the membrane diffusion and facilitated diffusion. The first part of this chapter concentrates on the energy-yielding catabolic processes: catabolic pathways of mono-, di- and polysaccharides in addition to some special topics like catabolism of polysaccharides of the plant structures and animal tissues. Further paragraphs cover the breakdown of lipids, fatty acids and special hydrocarbons. The energy-yielding process in the tricarboxyl cycle, including its regulation, casual overproduction, electron transport and its connection with the membrane structure is presented excellently. Other pathways like anaerobic oxidation with the special byproducts, photosynthesis, bioluminescence, CO_2 fixation as well as oxidative utilization of some special anorganic compounds are all thoroughly discussed. Further paragraphs deal with proteolysis, amino acid breakdown and other topics.

The second part of this chapter — biosynthesis — describes the synthetic pathways of basic cell constituents including the biosynthesis of vitamines and coenzymes. Short paragraphs represent the synthesis of pigments, antibiotics, mycotoxins and alcaloids. Among the biosynthetic processes of macromolecular compounds the replication of DNA, synthesis of RNA molecules and the

transcription and translation mechanisms can also be found. The biosynthesis of cell wall polysaccharide is mentioned shortly. There is a good but somewhat simplified summary of the regulation processes.

Chapter 5 — *Physiology of bacterial growth* — appears in a rather short summarized form.

Chapter 6 is entitled *Genetics*. The interpretation of genetical phenomena concerning bacteria is rather short. In the opinion of the reviewer this chapter is shorter than it should be. Topics like the mechanism of suppression, plasmids, the different mechanisms of recombination, the IS elements, transposons, etc. cannot be found in it. A short summary of the gene cloning might also be useful. The descriptions though in a simplified form are correct except for a few cases (e.g. generalized transduction).

Chapter 7 is a short summary of systematics of bacteria, viruses and fungi. Chapter 8 gives some selection of the applications of general microbiology (industrial production, vaccines, immune sera, microbial control of environment, etc.).

The book ends with a list of literature including important textbooks and special reviews for each chapter. Subject index is also attached. The schematic figures are amply used and are in excellent quality.

The book *General Microbiology* by *Weide* and *Aurich* is a useful up-to-date book for students or everyone interested in general microbiology. As mentioned before the proportion of some topics may be disputed, but this, in itself, is a rather subjective view. The chapter on metabolism is an especially good source of information for research work too. Another excellent feature of this book is that the very basic facts are also discussed and the modern concepts are brought forward with skillful teaching experience.

J. KÉTYI

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1980. II. 12. — Terjedelem: 7,35 (A/5) ív, 6 ábra

80.7985 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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HU ISSN 0001—5253 Index: 26.030

26. IX. 1980

Acta biochimica et biophysica

VOLUME 15
NUMBER 3
1980

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

ABBPAP 15 (3) 165–257 (1980)

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 165–172 (1980)

Preparation of a Target Specific Fraction Controlling the Proliferation of Granulocytes.

The Abolishment of the Specificity by Glutathion

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(Received October 20, 1979)

A low molecular weight fraction (GI-3) was prepared from peripheral horse blood leukocytes. This fraction inhibits the proliferation of bone marrow cells, the effect is dose dependent. The proliferation of thymocytes and HeLa cells is not affected by GI-3. This specifity was abolished when glutathion was applied together with GI-3: the ^{3}H-TdR uptake of thymocytes in vitro was completely inhibited, however, slight or no effect was observed on bone marrow and HeLa cells, respectively.

Introduction

In previous experiments we demonstrated that among three constituents of the mitodepressive extract of granulocytes prepared from rat and rabbit peritoneal exudate and peripheral horse blood fraction GI-1 (MW $5-10\times10^4$) exhibits an oligospecific inhibitory character (Balázs et al., 1972, 1975, 1977; Blazsek et al., 1976). A similar specific small molecular weight fraction was partially purified by Rytömaa and Kiviniemi (1968a, b), Paukovits (1971), Rytömaa et al. (1976) and Paukovits and Hinterberger (1978). In order to obtain a larger amount of the active components horse blood leukocytes were used instead of rat or rabbit peritoneal exudate as source of the inhibitors. The fraction GI-3 was further purified by paper electrophoresis and among its components glutathion was identified.

This paper describes the large scale preparation of GI-3 from horse blood leukocytes, and the abolishment of its target specificity by glutathion.

Materials and methods

Preparation of the crude extract (GI-3)

Peripheral horse blood was used as the source of mature granulocytes. 20 l fresh blood treated with heparin (3750 U/l, Richter, Budapest) divided in 2 l portions was allowed to sediment spontaneously at room temperature for 30

Abbreviations: GI-3, granuloid inhibitor fraction 3; GCE, granulocyte crude extract; sAML, subacute myeloid leukaemia; sALL, subacute lymphoid leukaemia; GSH, glutathion

minutes. The leukocyte rich upper layer (about 800 ml/2000 ml horse blood) was collected and centrifuged ($500 \times g$, for 5 minutes, at $+4$ °C) the supernatant was discarded. The cell mass was washed ($500 \times g$, for 5 minutes, at $+4$ °C) in phosphate buffer (pH 7.4, 0.06 M) by this procedure 3.3×10^9 leukocytes of 80% granulocyte purity was obtained from 1 l full blood. Washed granulocytes were disrupted in teflon lined Potter homogenizer and centrifuged by 1 500 g for 30 minutes at $+4$ °C. The supernatant was sieved through an Amicon PM 10 membrane (in ice bath, cut off limit at 10 000 daltons) and the filtrate was lyophilized. The lyophilisate was dissolved in distilled water and the precipitate centrifuged ($10\,000 \times g$). The supernatant was chromatographed on Sephadex G-10 (v_t = 400 ml) eluted with ammonium bicarbonate (0.05 M, pH 7.9, at a flow rate of 40 ml/h). The absorbance of fractions were continuously recorded at 280 nm and 225 nm and tested for inhibitory action. The collected fractions were lyophilized.

Carboxymethylation

5 mg of GI-3 fraction was dissolved in 0.2 ml ammonium bicarbonate (0.1 M pH 7.9). The solution was incubated with 0.2 μmol dithioerythrit for 30 min at room temperature. After the incubation 0.3 μmol ^{14}C bromacetate was added, the pH of the reaction mixture was readjusted if necessary. The whole reaction mixture was directly applied to 1.5×50 cm Sephadex G-10 column, previously equilibrated with 0.1 M ammonium bicarbonate buffer, pH 7.9 at 20 ml/h flow rate. Two ml fractions were collected, the radioactivity was monitored by Packard-Tri-Carb scintillation counter.

Electrophoresis

The freeze-dried sample was dissolved in distilled water, applied to Whatman 3 MM electrophoresis paper. The separation of the components was performed by two dimensional high voltage electrophoresis at pH 6.5 (pyridine–acetic acid–water 90 : 4 : 900 by vol.), and pH 1.9 (acetic acid–formic acid–water 8 : 2 : 90 by vol.). The radioactive compounds were located by autoradiography.

Amino acid analysis

The radioactive component was eluted with 0.05 M ammonium hydroxide freeze dried and hydrolized with 6 N HCl, at 105 °C, 16 hours. The amino acid composition was determined by Beckman-Unichrom analyzer.

Determination of the inhibitory activity

The collected fractions were tested in the following assay systems to determine their biological activity and specificity. Bone marrow cells from the femora of male Wistar rats of 40 g body weight were suspended in TC-199 medium at a cell density of 3×10^6/ml, and cultivated at 37 °C. The thymocyte suspension

cultures were prepared using the same animals by cutting the thymus into tiny pieces and agitated with forceps. The larger pieces were removed. Cells were cultured also in TC-199 medium at 5×10^6/ml density.

HeLa S3 cells were permanently sustained in Falcon plastic dishes in TC-199 medium complemented with 10% ABO serum. The logarithmically growing cells were passed into Belco tubes (1.5×10^5 cell/ml) for about 20 hours. Before testing the cells were washed in TC-199 medium three times to remove the serum. Subacute myeloid and lymphoid leukaemic rats were sacrificed on the 12–14th and 15–17th post-transplantation days, resp. Suspension cultures from the bone marrow and thymocytes of these animals were prepared as from the normal animals. These transplantable leukaemias were originally induced by 7,12-dimethyl-benzanthracene. The transplantation was carried out by 6×10^4 spleen cells injected into the peritoneal cavity of 2 to 4 day old rats (Gál et al., 1973).

The proliferation rate of the suspension cultures was measured by ^{3}H-TdR incorporation into the acid-insoluble DNA. The pulse label (1 μCi/ml with a specific activity of 19.5 mCi/mM, UWVR, Prague, Czechoslovakia) was used at 3 to 4 hours. Radioactivity incorporated into acid insoluble DNA was determined by filter disc method (Chou et al., 1974; Blazsek, Gaál 1978). The aliquots (100 μl) from each culture were pipetted onto Whatman 3 MM paper discs. After having dried they were plunged into ice cold 5% $HClO_4$ for 2×5 min and twice into ethanol and diethylether (for 5 min in each case). The radioactivity of washed and dried discs was measured in toluene-based cocktail in Packard-Tri-Carb liquid scintillation spectrometer.

Experimental result

The low molecular weight (> 10 000) cytosol extract of purified granulocytes was chromatographed on Sephadex G-10. All fractions were tested on bone marrow cultures. The biologically active fractions range: $v_e/v_o = 1.5-1.45$ were collected and called granuloid inhibitor-3 (Balázs et al., 1977). 130 mg GI-3 was prepared from 20 l horse blood. At 500 μg/ml concentration the granuloid inhibitory fraction decreased ^{3}H-TdR incorporation into acid insoluble DNA of bone marrow suspension cultures to 30–60% of the normal value (15 tests with different preparation batches). Thymocytes, however, were only inhibited by 0–15%. The dose-response relationship of the inhibitory effect of GI-3 on bone marrow cells is represented on Fig. 1, and on thymocytes on Fig. 2.

The specificity of the active material was tested on HeLa S3 cells and thymocytes of sAM and sAL leukaemic animals. These experiments resulted in slight if any effect of GI-3 on the ^{3}H-TdR incorporation of these non-myeloid cells.

The stability of GI-3 was also tested. These experiments showed that the active component of GI-3 does not lose its activity in TC-199 medium at 37 °C for 72 hours. The lyophilized GI-3 can be stored for months at −20 °C. Chromatography of the cytosol extract, was performed at +4 °C to active GI-3.

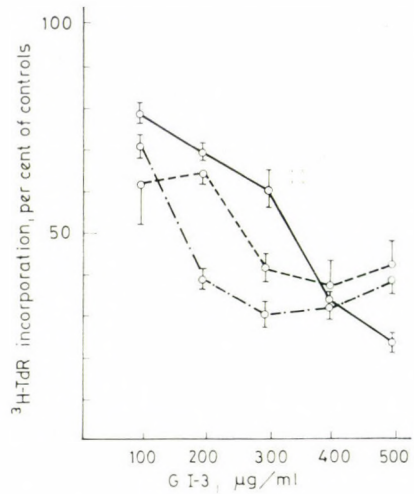

Fig. 1. The dose-response effect of GI-3 and its synergism with -SH compounds on bone marrow cells. ^{3}H-TdR incorporation was determined by pulse label at 3—4 hours. The standard errors were calculated from the average of 6 measurements
GI-3: ———, GI-3 + 0.325 mM GSH (100 μg/ml): —·—·—, GI-3 + 0.325 mM L-cystein: — — —

GI-^{3} synergism with glutathion

Glutathion was detected in the carboxymethylated and rechromatographed GI-3. This fact is in accordance with the high GSH level of cytosol extract of leukocytes and other cells (Kosower, Kosower, 1978). After the carboxymethylation we could isolate a ^{14}C labelled compound by gel chromatography and two dimensional paper electrophoresis. The N-terminal group of this compound was Glu, the acid hydrolysate showed the presence of $Glu_1CmCys_1Gly_1$: According to this finding this peptide was identified as glutathion.

The effect of GSH on thymocytes is shown on Fig. 3. Similarly, there is no effect on bone marrow cells. An about 30% increase in the inhibition of GI-3 used in 200 or 300 μg/ml concentration can be detected by adding 100 μg/ml GSH to the bone marrow cultures (Fig. 1), the ED_{f_0} of GI-3 decreasing from 340 to 185 μg/ml. This synergic effect of GSH is more surprising measured on thymocytes, 300 μg/ml GI-3 causing nearly 100% inhibition applied together with 100 μg/ml GSH (Fig. 2). The GSH content of GI-3 is about 1/1000 part of its dry weight. The concentration dependent effect of GSH was also studied (Fig. 3). Increasing concentration of GSH potentiated the effect of 500 μg/ml GI-3, an about 30 μg/ml GSH caused 50% inhibition in the ^{3}H-TdR incorporation of thymocytes calculated from Fig. 3. The next step was to examine the synergic action of GI-3 and GSH on thymocytes and bone marrow cells of sAML and sALL rats Fig. 4.

In these cases the cells were labelled at the 0 to 2 hours. Pulse labelling at the 3 to 4 hours resulted in nearly 100% inhibition of leukaemic thymocytes. The role of -SH group in this synergism was studied by testing the effect of GI-3 and L-cystein. This reducing agent (0.325 mM) slightly potentiates the effect of 300 μg/ml GI-3 on bone marrow cells (Fig. 1). On thymocytes 0.325 mM of L-cystein caused a similar effect than GSH did (Fig. 2), but did not influence the ^{3}H-TdR incorporation if the compound was applied alone.

Discussion

There is an increasing body of evidence that the granulocyte proliferation is controlled by endogenous factors. Many data render the multifactorial regulation of this process probable (Cline, Golde, 1979a, b). Despite this, neither the colony stimulating activity (Broxmeyer, 1978), nor the putative granulocytic chalone have yet been isolated in chemically pure form although such work is in progress in several laboratories (Paukovits, Hinterberger, 1978; Rytömaa, Toivonen, 1979).

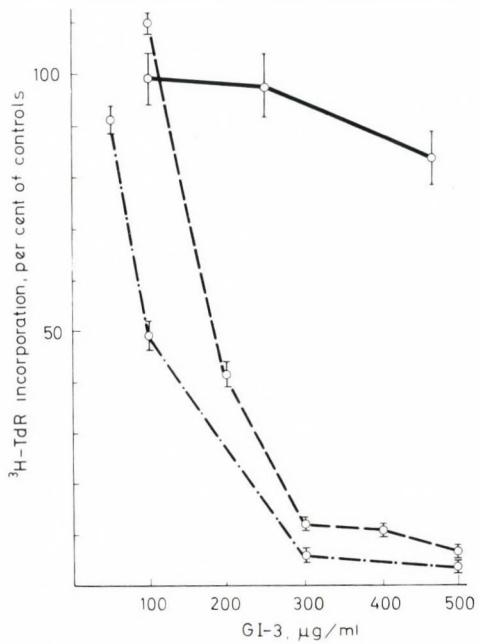

Fig. 2. The synergic effect of different amounts of GI-3 and GSH on thymocyte cultures. ^{3}H-TdR incorporation was determined by pulse label at 3—4 hours. The standard errors were calculated from the average of 6 measurements
GI-3: ———, GI-3 + 0.325 mM GSH: —·—, GI-3 + 0.325 mM L-cystein: — — —

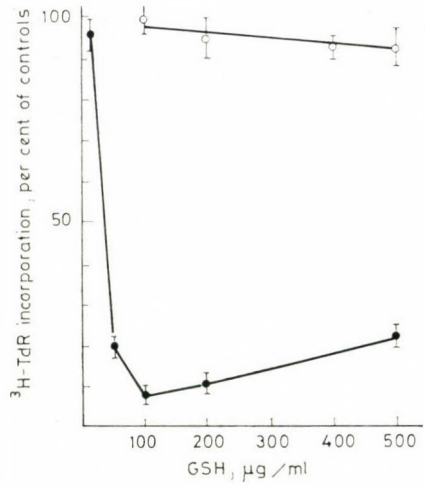

Fig. 3. The synergic effect of GI-3 applied together with different concentrations of GSH on thymocytes. ^{3}H-TdR incorporation was determined by pulse label at 3—4 hours. The standard errors were calculated from the average of 6 measurement
500 µg/ml GI-3 + GSH: —●—, GSH: —○—

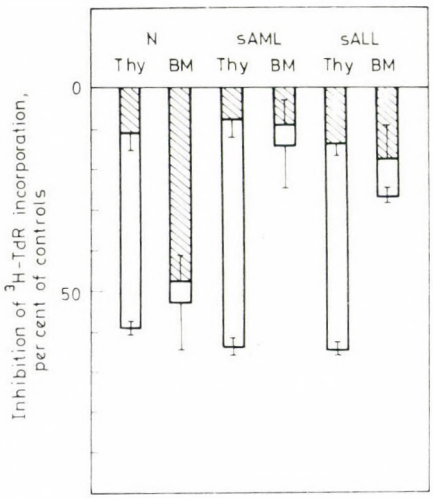

Fig. 4. The synergic effect of GI-3 and GSH on different normal and leukaemic bone marrow and thymocyte cultures. N: normal, sAML: subacute myeloid leukaemia, sALL: subacute lymphoid leukaemia, Thy: thymus, BM: bone marrow, the hatched part of the columns represents the effect of 500 µg/ml GI-3 on the indicated tissue cultures. The whole part of the columns represents the effect of 500 µg/ml GI-3 added with 0.325 mM (100 µg/ml) GSH. ^{3}H-TdR incorporation was determined from 0 to 2 hours

One of the main problems is to obtain partially purified material enough to the chemical purification and chemical characterization. Various sources and ways were used to prepare crude extracts having the properties of granulocytic chalone (Aardal et al., 1977). In our experiments the peripheral horse blood proved to be a very promising source to obtain large amount of endogenous inhibitor of granulopoiesis, due to the rapidity and productivity of the procedure.

A low molecular weight inhibitory fraction, GI-3 was prepared from the extract of horse peripheral granulocytes. The molecular weight of the active component amounts to approximately 600.

Similar crude factor was prepared by Paukovits et al. (1977). The target cells of GI-3 are the immature granulocytes, the inhibitory effect is dose dependent. This effect is specific, since it has influence neither on thymocytes nor on HeLa S3 cells.

The examination of GSH on our culture systems was motivated by the following facts:

1. GSH is a component of GI-3.

2. GSH plays an important role in a large number of biological functions (Kosower, Kosower 1978; Meister, Tate 1976), so it may have influence in granulopoiesis.

3. The concentration of GSH is 3.5–5 mM in leukocytes.

The ineffectiveness of GSH on bone marrow cells and thymocytes ruled out the possibility that the effect of GI-3 is due to its GSH content. GI-3 applied together with GSH, however, resulted in a striking effect on both normal and leukaemic thymocytes. The slight effect experienced on bone marrow cells can be attributed to the lymphocyte content of the marrow.

Synergic effect was not found on HeLa cells. To explain the synergism on non target thymocytes some possibilities arise:

- GSH may specifically change the redox-state of the cells. L-cystein caused a similar effect on thymocytes than GSH did.
- GSH may influence the activity of the membrane receptors of lymphocytes which play a part in the cell proliferation or in the thymidine uptake of the cells.
- One of the component of GI-3, may be either the real regulator of the cell proliferation or having different activity, forms a complex with GSH and with the contribution of gamma-glutamyl-transpeptidase may enter the cell (Jones et al., 1979), where presumably its activity on the cell proliferation is exerted.

This latter idea is supported by the facts that gamma-glutamyl-transpeptidase can be found on the surface of lymphocytes (Novogrodsky et al., 1976), and its inhibitor, serine borate buffer of 0.5 mM decreased the effect of 500 μg/ml GI-3 and 100 μg/ml GSH by 26.9% on thymocytes.

The effect of L-cystein, however, can not be explained by this idea.

To prove the intracellular action of the active component of GI-3 further investigations are needed, using chemically identified and labelled material.

The authors wish to thank to Mr L. Csáky for radiochemical measurements, to Dr F. Gál who kindly offered the leukaemic rats and to Mrs K. Barabás and Mr J. Fitos for their assistance.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 173–176 (1980)

A Rapid Thin Layer Chromatographic Method for the Quantitative Determination of Hydroxyproline and Hydroxylysine

(Short Communication)

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(Received December 3, 1979)

There are a number of well established methods for the thin layer chromatographic analysis of the twenty common standard amino acids found in proteins. Combination of ion exchange TLC separation with evaluation by video-densitometry offers quick and suitable procedures for quantitative routine analysis (Dévényi et al., 1971 a; 1971 b; Ferenczi et al., 1971). Such simple methods, however, are not available for the determination of the "rare" amino acids, which are characteristic components of certain proteins. Hydroxyproline (Hyp) and hydroxylysine (Hyl) belong to such amino acids. They occur in relatively high quantities in collagen. In this paper we describe a new method for the rapid and quantitative determination of these two amino acids.

1. *Determination of hydroxylysine*

Precoated chromatosheets, type Fixion 50×8, size 20×20 cm (Chinoin), were used in Na^+ form for the separation. The buffer used for elution had the following composition (Dévényi et al., 1972):

citric acid monohydrate:	24.6 g
sodium hydroxide:	14.0 g
hydrochloric acid 37%:	6.5 ml

The solution was made up to 1000 ml with deionized water and the pH was adjusted with sodium hydroxide to 5.28 instead of 5.23 described for amino acid separation by Dévényi et al. (1972). We have found a better separation of Hyl from Lys at this slightly less acidic pH.

The chromatography was performed at room temperature, the run required about 3 hours. The sheets were air-dried and sprayed with the following staining reagent:

50 ml of solution A + 20 ml of solution B + 30 ml of distilled water. Solution A contained 2 g of ninhydrin in 100 ml acetone and solution B 1 g of Cd-acetate in 50 ml glacial acetic acid + 100 ml of distilled water.

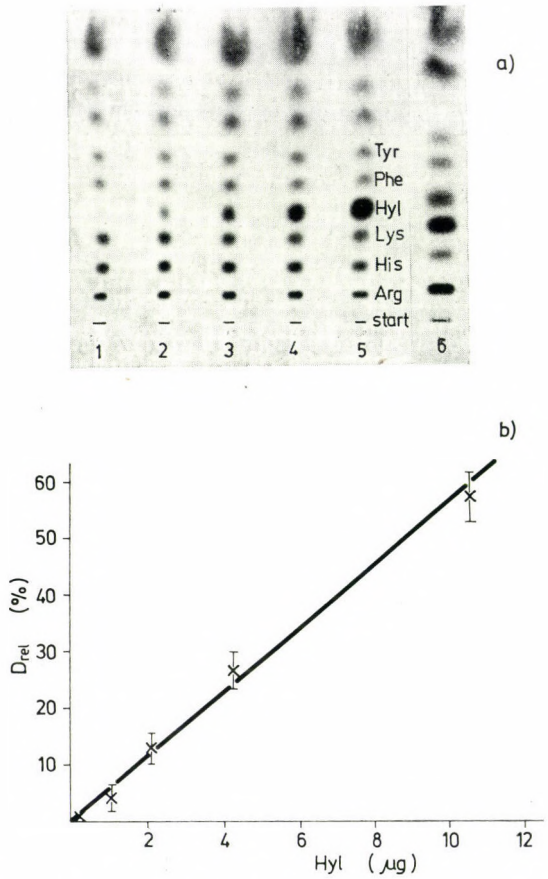

Fig. 1. Chromatographic determination of Hyl. a.) One dimensional separation of Hyl-containing amino acid mixtures (1—5) and an acidic hydrolysate of pigskin (6), in sodium citrate buffer, pH 5.28, on Fixion 50× 8. The 4 μl samples applied to the sheet contained 10 nmol of each standard amino acids and 0.211 (1) 1.055 (2) 2.11 (3) 4.22 (4) and 10.55 (5) μg Hyl. b.) Video-densitometric calibration curve showing the relative densities (D_{rel}) as a function of the amounts of Hyl applied in the samples. The standard deviations of the values are also indicated (n = 8)

After staining, the chromatosheets were allowed to stand overnight at room temperature in the dark, then evaluated by video-densitometry (Telechrom OE-976) in a horizontal way. This mode of evaluation can be achieved if the plates are rotated 90 degrees before being placed in the instrument (Csorba et al., 1979). In this way those spots which migrate at identical R_f values in parallel runs can be compared.

Figure 1a shows the chromatogram of an amino acid mixture containing hydroxylysine. Hyl migrates as a well separated spot between Lys and Phe. This

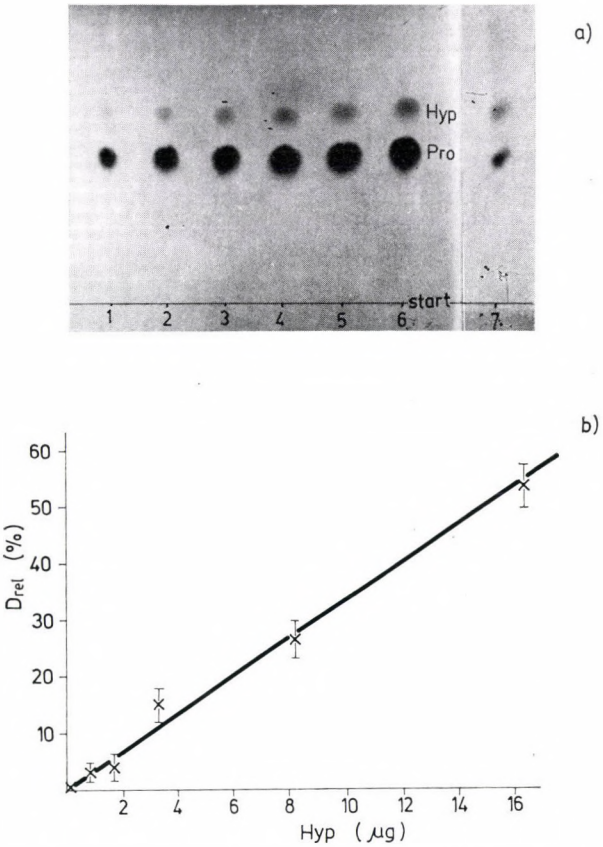

Fig. 2. a.) One dimensional separation of Pro and Hyp mixtures (1—6) and an acidic hydrolysate of pigskin (7), on silica gel G layer, using a solvent mixture of i-propanol — water (7 : 3) containing 0.1% isatin and 0.2% p-dimethylamino-benzaldehyde. The quantities of Hyp applied to the plate were 0.16 (1) 0.81 (2) 1.63 (3) 3.27 (4) 8.15 (5) and 16.3 (6) μg b.) Calibration curve for Hyp by video-densitometric measurements, indicating also the standard deviations (cf. Fig. 1b)

method is useful for the determination of Hyl if there is no ornithine in the sample. This latter rare amino acid also migrates between Lys and Phe (Hrabák, Ferenczi, 1971), however, collagen and other proteins do not contain ornithine.

Figure 1b shows the densitometric calibration curve for the quantitative evaluation of Hyl. The best range for the determination is about 0.5—5 μg of Hyl in a spot.

Since the hydrolyzates of proteins in general do not contain such high amounts of Hyl, it seems to be advantageous to add 2 μg standard Hyl to the samples and to determine the increase in D_{rel} as compared to a control spot containing the standard Hyl alone.

2. *Determination of hydroxyproline*

For the separation of Hyp home-made silica layers were used. For preparing TLC plates 25 g of silica gel G (Reanal) was suspended in 60 ml water and the resulting slurry was spread with a Desaga spreader on the surface of 20 × 20 cm glass plates to a thickness of 0.25 mm. The wet plates were air dried at room temperature over night. The chromatographic run was carried out at room temperature using the following solvent system: i-propanol-water (7 : 3) containing 0.1% isatin and 0.2% p-dimethylamino-benzaldehyde. It was advantageous for the reproducibility of the quantitative measurements to dissolve the staining reagents in the solvent mixture. After about two hours run the chromatoplates were heated at 80 °C for 15 minutes. By this time the spots of proline appeared. Following this, a second incubation in hydrogen chloride vapour at 80 °C resulted in the appearance of the violet colour of the Hyp spot.

Figure 2 shows the chromatogram of a mixture of Pro and Hyp and the densitometric calibration curve for the quantitative evaluation of Hyp. The quantity of Pro and Hyp can be measured selectively because they migrate as well separated spots. The difference in R_f values of the two amino acids was 0.14 ($R_{f\,Pro} = 0.43$, $R_{fHyp} = 0.57$). This difference is more than that published earlier by Brenner and Niederwieser (1960). These authors used a n-propanol–water (7 : 3) solvent mixture. We have found that use of i-propanol instead of n-propanol resulted in a better separation. The presence of other amino acids does not influence the analysis because they do not give colored spots under the conditions described above. At higher temperatures or during prolonged incubation other amino acids also react with isatin and disturb the quantitative estimation of Hyp.

Our method seems to be suitable for the rapid routine quantitative estimation of Hyp in the range of 1.0–15.0 μg per spot.

Quantitative determinations of Hyp and Hyl contents in protein hydrolysates will presumably become a useful tool in food qualification.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 177–183 (1980)

Changes in the Intramembrane Viscosity of Sheep Erythrocytes in the Presence of Membrane Bound Proteins

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(Received November 3, 1979)

Some dynamic properties of protein- and antibody-treated cell membranes were investigated. The membrane viscosity of sheep erythrocytes increased upon binding various proteins as determined from the fluorescence emission anisotropy of 1,6-diphenyl 1,3,5-hexatriene dissolved in the membranes. However, specific, IgM type immunoglobulins produced against the erythrocytes decreased the intramembrane viscosity of cells.

The applicability of the above dye to follow the binding of specific and non-specific proteins to erythrocytes is also discussed.

Introduction

Fluorescence polarization degree of DPH has widely been used to monitor the physical states of lipid bilayers and cell membranes (Shinitzky, Barenholz, 1974; Shinitzky, Inbar, 1974; Andrich, Vanderkooi, 1976; Shinitzky, Inbar, 1976; Kinosita et al., 1977). Membrane fluidity is highly dependent upon the cholesterol to phospholipid ratio, the sphingomyelin content and the amount of unsaturated fatty acids (Inbar et al., 1974; 1977; Barenholz et al., 1976; de Laat et al., 1977; Blitterswijk et al., 1977; Shinitzky, 1978; Cooper et al., 1978). Membrane structure is strictly controlled by cell metabolism and the conformational (e.g. free or bound) states of membrane receptors which are mostly proteins. An increase in protein content can immobilize the membrane (Cornell et al., 1978).

The present paper deals with the changes in membrane fluidity induced by addition of specific antibodies and other, nonspecific, proteins as monitored by DPH. Specific antibodies decreased the membrane viscosity. Various nonspecific proteins, on the contrary, increased the rigidity of intramembrane environment.

Materials and methods

Freshly prepared sheep red blood cells (or in some cases cells stored at 4 °C for some days) were mixed in a 1 : 1 ratio with Alsever buffer and a 2.5% cell-suspension was made in PBS, pH 7.2. This suspension was further washed with

Abbreviations: DPH, 1,6-diphenyl 1,3,5-hexatriene; PBS, phosphate buffered saline.

PBS, centrifuged at 800 x *g* for 10 minutes. Then the suspension was diluted by PBS to 10^7 cells/ml. The haemolysin (Human, Budapest, Hungary) was produced in rabbits against sheep erythrocytes. The titer was obtained from complement fixation probes. The C 50 titer was found to be $1 : 4 - 500$.

Globulins were prepared from bovine sera by ammonium sulfate precipitation. The precipitate was washed several times with PBS. After dialysis against isotonic NaCl the globulin fraction was purified by passing it through a column of DEAE-Sephadex A-50 (1.8×20 cm) and elution with 0.2 M phosphate buffer, pH 6.4.

Preparation of IgM: The globulins were precipitated by saturated $(NH_4)_2SO_4$ from immunosera produced against sheep erythrocytes in rabbits. The precipitate was washed twice with saturated $(NH_4)_2SO_4$ and dialyzed against distilled water for 24 hours. After a subsequent short dialysis to equilibrate the solution with 0.05 M TRIS-HCl, pH 7.0, the IgM fraction was prepared by the use of a Sephadex G-200 (2×30 cm) column. Immunoglobulins were collected in a fraction collector and classified by immunoelectrophoresis according to Scheidegger's micromethod (Scheidegger, 1955). Protein content was determined by the Lowry method (Lowry et al., 1951).

Treatment of cells with sera and immunoglobulins: When specific serum was applied, it was diluted 50 to 1000 fold and 1 ml aliquots of the dilutions were mixed with 1 ml erythrocyte suspensions (10^7 cells/ml). After 60 min of incubation at 37 °C the cells were washed twice with PBS solution.

Samples were similarly prepared with cells and bovine globulins containing $0.1 - 0.8$ mg/ml protein or $10 - 100$ μg/ml IgM.

Fluorescence labeling of cells: DPH Sigma Chemical Company, was used as a probe for monitoring the fluidity of membrane lipids (Shinitzky, Inbar, 1974; Inbar et al., 1974). A solution of 2×10^{-3} M DPH in tetrahydrofuran was first diluted 1000-fold in PBS, pH 7.2. Stirring was continued for 100 minutes at room temperature. The clear dispersion of 2×10^{-6} M DPH had practically no fluorescence. One volume of DPH dispersion was mixed with one volume of cell suspension (10^7 cells/ml) and incubated for 30 minutes at 37 °C.

Fluorescence anisotropy analysis: Steady state emission anisotropy was measured in a Hitachi MPF-4 fluorescence spectrophotometer equipped with polarizers. Thermostated (37 °C) 300 μl silica cells were used for measurements performed immediately after completing the preparative work and treatments. In order to reduce experimental errors due to the scattered light at fluorescence polarization measurements (Shinitzky et al., 1973), cell concentrations were kept below 5×10^5 cells/ml (Inbar et al., 1974).

Fluorescence anisotropy (r) was calculated from the following equation:

$$r = \frac{I_{VV} - G\, I_{VH}}{I_{VV} + 2\, G\, I_{VH}} \tag{1}$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with vertical and horizontal analyzers, respectively. The polarizer was in vertical position in both cases.

$G = I_{HV}/I_{HH}$ is the correction factor. The first character of the indices in equation (1) denotes the position of the polarizer and the second that of the analyzer.

Microviscosities were calculated according to the Perrin equation (1934):

$$\frac{r_o}{r} = 1 + C(r)\frac{T\tau}{\eta} \tag{2}$$

where r_o = the limiting fluorescence anisotropy having the value of 0.362 (Shinitzky, Barenholz, 1974), r = the measured fluorescence anisotropy, τ = the excited state lifetime of DPH, η = the microviscosity of the medium in which the DPH molecules are embedded, C(r) = the parameter which relates to the molecular shape of the fluorophore and has a specific value for each r (Shinitzky, Inbar, 1974). Its value is 8.6×10^4 Pa.s deg.$^{-1}$s^{-1} (Shinitzky, Inbar, 1974).

The value of τ for DPH at 37 °C was estimated indirectly to be 9.7 nsec from the temperature profile of the fluorescence intensity determined by Shinitzky and Barenholz (1974).

The changes in microviscosity (η) with that of the temperature were described as

$$\eta = A \exp \frac{\Delta E}{RT} \tag{3}$$

where E is the activation energy calculated from the plot of ln η vs. 1/T.

Results

Table 1 shows the anisotropy and calculated microviscosity values for control and immunoserum-treated control sheep erythrocytes. The eleven independent experiments clearly demonstrate that both the anisotropy and the microviscosity of the immunoserum-treated cell membranes are lower than those of the controls. As the amount of the immunoserum was gradually decreased the changes in anisotropy and microviscosity also decreased as shown in Table 2. In order to learn more about the effect of membrane-bound proteins on membrane fluidity, experiments were carried out with different control sera of several species (Table 3). The erythrocytes showed a highly significant increase in anisotropy and microviscosity, i.e. an increase in membrane rigidity. This increase slightly differed in its extent, depending on the origin of the sera. To explore the mechanism of membrane viscosity changes, their temperature dependence was also investigated. A typical temperature dependence of membrane viscosity is shown in Fig. 1 after treating the erythrocytes with either immuno- or normal sera. The ln η was linearly dependent on T^{-1} between 21–37 °C, suggesting that significant changes in the lipid phase were not likely to occur.

In order to exclude the possibility that the different lipid components of the full sera might interfere with the DPH – erythrocyte interaction, experiments similar to those demonstrated in Tables 1–3 were carried out also with purified

Table 1

Anisotropy (r) and calculated microviscosity (η) values for control and immunoserum-treated sheep erythrocytes

(t = 37 °C)

Exp. No.	Sheep erythrocytes			
	treated with immunoserum[a]		control	
	r	η^b (Pa · s)	r	η^b (Pa · s)
1.	0.178	0.250	0.200	0.319
2.	0.215	0.378	0.222	0.410
3.	0.208	0.349	0.217	0.387
4.	0.218	0.391	0.217	0.387
5.	0.212	0.365	0.234	0.473
6.	0.213	0.370	0.217	0.387
7.	0.203	0.330	0.225	0.425
8.	0.180	0.256	0.238	0.496
9.	0.175	0.242	0.221	0.405
10.	0.174	0.239	0.203	0.330
11.	0.200	0.319	0.220	0.400
Mean:	0.198	0.317	0.219	0.402
SD	±0.005	±0.018	±0.003	±0.016

[a]: The specific immunoserum produced against sheep erythrocytes in rabbits was diluted 1 : 100.

[b]: Calculated values (for details see Materials and Methods).

Table 2

Effect of immunoserum[a] on the anisotropy (r) and microviscosity (η) of sheep erythrocyte membranes

(t = 37 °C)

Serum dilution	r	η^b (Pa · s)
1 : 50	0.168	0.238
1 : 100	0.179	0.268
1 : 500	0.187	0.294
1 : 1000	0.189	0.298
none	0.200	0.339

[a]: Immunoserum was produced against sheep erythrocytes in rabbits.

[b]: Calculated values (for details see Materials and Methods).

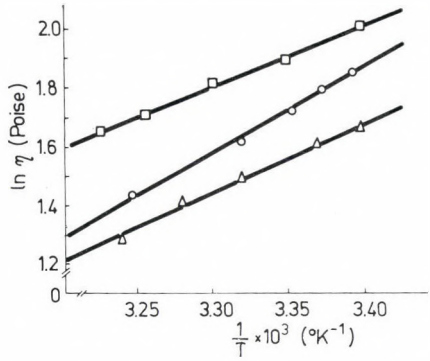

Fig. 1. Temperature dependence of the microviscosity (η) of DPH-labeled sheep erythrocyte membranes. Control (— o— o); after treatment with bovine globulin (— □—) or haemolysin (— △—)

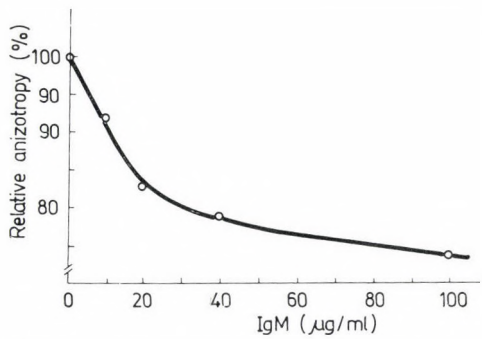

Fig. 2. Relative changes at 37 °C in the anisotropy of DPH embedded in IgM-treated sheep erythrocyte membranes as a function of the IgM concentration. The 100% value corresponds to 0.272

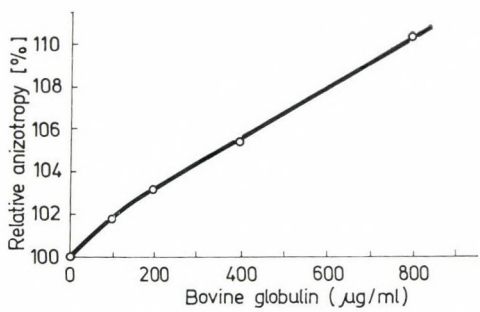

Fig. 3. Relative changes at 37 °C in the anisotropy of DPH embedded in bovine globulin-treated sheep erythrocyte membranes as a function of the globulin concentration. The 100% value corresponds to 0.221

Table 3

Effect of normal sera from different animals on the anisotropy (r) and microviscosity (η) of sheep erythrocyte membranes

(t = 37 °C)

The origin of normal sera (diluted 1 : 50)	r	η^a (Pa · s)
Rabbit	0.248	0.611
Rat	0.266	0.779
Bovine	0.256	0.685
None	0.222	0.447

[a]: Calculated values (for details see Materials and methods).

IgM immunoglobulins. Figure 2 shows that purified IgM also caused a decrease of anisotropy and, in accordance with the data presented in Table 3, bovine globulin increased membrane anisotropy (Fig. 3). These effects showed a similar concentration dependence as found with the full sera.

Discussion

Temperature dependent motility of different membrane constituents, usually termed membrane fluidity, has a significant regulatory role in cell metabolism (Singer, Nicolson, 1972) and also in transmembrane transport phenomena (Doonan, 1977). On the other hand, metabolic processes can also influence membrane fluidity and thus regulate the accessibility of membrane receptors.

According to our results the intramembrane viscosity of sheep erythrocytes is decreased by specific IgM immunoglobulins and increased by non-specific proteins. These observations are somewhat different from those of Nicolau and his co-workers who found an increased membrane fluidity of density-inhibited normal chicken fibroblasts stimulated by 10% bovine serum. However, cells from non-confluent or oncorna virus-transformed cultures did not show such an increase (Nicolau et al., 1978).

On the basis of the above, the following model is suggested to account for the observed phenomena: Non-specific proteins can be built into the lipid regions of the membrane. Thus the membrane becomes more rigid even if we disregard the likely compression of the remaining free lipid regions. However, the immunoglobulins bound to the surface antigens have quite a different effect. The uniformly distributed antigen receptors may form a more dense islet upon the binding of specific antibodies. Consequently, larger confluent lipid mosaics will show higher fluidity. Of course, the experimental model investigated in this communication has a limited validity because of the specific and unique properties of the IgM globulins.

Experiments with other cells, mostly human lymphocytes and different types of immunoglobulins are in progress in order to get a deeper insight into the mechanisms of the phenomena presented in this communication.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 185—198 (1980)

Physicochemical Characterization of the Cytosol Glucocorticoid Receptors in Various Lymphoid Tissues

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(Received October 15, 1979)

Cytosol glucocorticoid receptors of five different lymphoid tissues of chickens and rats were characterized and compared by physicochemical parameters.

Binding specificity of receptors was found to be similar, except that cytosol receptors of chick tissues appeared slightly more specific for dexamethasone than those from rats.

Association rate constants of the dexamethasone-receptor complex were higher by about one order of magnitude in the case of chicken thymus and bursa than in other cytosols. Dissociation of dexamethasone-receptor complex occurred in one step in rat tissues, whereas in case of chicken tissues two phases of dissociation were discernible. Differences between K_a and the ratio of the association and dissociation rate constants are explained by non-equilibrium conditions.

The stability of receptors from the two species differed significantly, the chick receptor being more stable.

Hormone-receptor complex from each tissue could be separated into two fractions by DE-52 chromatography. The peak eluted at the lower KCl concentration in the case of chick corresponded to the fast dissociating hormone-receptor complex.

Kinetic analyses proved to be just as useful as ion-exchange chromatography for the characterization of, and distinguishing between, glucocorticoid receptors.

Introduction

Proteins that bind glucocorticoids with high affinity and specificity were found in a wide variety of sources, among them different lymphoid tissues. Responsiveness to glucocorticoids does not seem to be correlated with the glucocorticoid receptor content of the cell (Duval et al., 1976; Norman, Thompson, 1977; Lydigsen, 1978; Venetianer et al., 1978, Crabtree et al., 1978). In a recent study we demonstrated that sensitivity of various lymphoid tissues and cells to glucocorticoid treatment did not change parallel with the number and association constant of their glucocorticoid receptor (Náray et al., 1980). Physicochemical characterization of glucocorticoid receptors from lymphoid tissues of the rat and the chicken was therefore undertaken to reveal possible differences in their properties that might explain the differences in response as reported elsewhere (Náray et al., 1980).

Our methods for receptor characterization are similar to those used for comparison of glucocorticoid receptors from rat adipose tissue, thymus and kidney (Feldman et al., 1978) and of receptors from polymorphonuclear and mononuclear

leukocytes (Murakami et al., 1979). However, because of the limitations of equilibrium methods (Arányi, 1979) a greater emphasis was given to kinetic measurements.

Materials and methods

Materials

(1,2-^{3}H)-dexamethasone[1] (16 Ci/nmole) was obtained from Amersham (U.K.). Non-radioactive dexamethasone was a generous gift of Merck Sharp and Dohme Research Labs (Rahway, N. J.). Other steroids were purchased from G. Richter Ltd. (Hungary). Bovine serum albumin from Sigma (USA), DE-52 ion exchanger was the product of Whatman (England). All other reagents were of reagent grade and obtained from Reanal, Budapest, Hungary.

Methods

Determination of dexamethasone binding. Cytosol was prepared and specific dexamethasone binding was determined as described (Arányi, 1979). All incubations were performed at 0° except when indicated otherwise.

Protein was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Competition experiments. Incubation of cytosol with 20 nM ^{3}H-dexamethasone was performed in the absence or presence of 10^{-7} M or 10^{-6} M unlabeled competing steroid at 0° for 180 min. Steroid stock solutions (10^{-2} M) were prepared in absolute ethanol, the ethanol concentration in the reaction mixture was less than 1%. Protein bound radioactivity was determined from 250 μl aliquots after charcoal treatment.

Determination of rate constants

a) Dissociation rate constant (k_{-1}). The cytosol was made 20 nM with respect to ^{3}H-dexamethasone and 2 h was allowed for association to occur. Then unlabeled dexamethasone was added to a final concentration of 5×10^{-6} M (zero time). 100 μl aliquots were taken at different times to determine protein bound radioactivity after removing the free hormone by charcoal adsorption. No correction was made for nonspecific binding which was always found to be less than 10 per cent. Data were analyzed from semilogarithmic plots. When two phases

[1] Systematic names of steroids used: Aldosterone: 11β,21-Dihydroxy-18-al-4-pregnene-3,20-dione; Cortexolone: 17α,21-Dihydroxy-4-pregnene-3,20-dione; Corticosterone: 11β,21-Dihydroxy-4-pregnene-3,20-dione; Dexamethasone: 9α-Fluoro-16α-methyl-11β-,17α,21-trihydroxy-1,4-pregnadiene-3,30-dione; Hydrocortisone: 11β,17α,21-Trihydroxy-4-pregnene-3,20-dione; Estradiol: 1,3,5(10)Estratriene-3,11-diol; Progesterone: 4-Pregnene-3,20-dione; Testosterone: 17α-Hydroxy-4-androstene-3-one; Triamcinolone acetonide: 9α-Fluoro-11β,-16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide.

were observed, data of the first phase were corrected for extrapolated values of the second phase.

b) Association rate constants (k_1). To determine the rate constant of hormone-receptor complex formation, the cytosol was mixed at zero time with an equal amount of buffer containing $5 \times 10^{-8} - 2.5 \times 10^{-7}$ M ^{3}H-dexamethasone. The concentration of the labeled hormone was chosen such that association rate could be conveniently followed in the case of each tissue. Aliquots were taken at different times to determine protein bound radioactivity. Corrections were made for nonspecific binding by the use of similar reaction mixtures which contained an additional 100 fold excess of unlabeled dexamethasone. From the total amount of hormone receptor, $[H_0]$ and $[R_0]$, respectively, concentration of free hormone, [H], and free receptor, [R], was calculated. Since back reactions were negligible in each case,

$$\ln \{([H]/[R])/([H_0] - [R_0])\}$$

vs. t plots gave straight lines with a slope of k_1. $[R_0]$ was estimated from the association constant reported in (2), and from [H(t)] and [R(t)] values determined at t = 2.5 h.

c) Denaturation rate constant (k_2). The cytosol was prepared in 0.01 M Tris-HCl buffer, pH 7.4, containing 10% (v/v) glycerol, 1.5 mM EDTA, 2 mM DTT, saturated with N_2, and was treated as described above. Aliquots were taken at different times to determine specific ^{3}H-dexamethasone binding.

The parameters of the straight lines obtained in all cases (a, b, c) were estimated by the method of least squares and correlation coefficients were higher than 0.98.

Ion exchange chromatography. Minicolumns (1.2 × 2.5 cm, 2.8 ml bed volume) of DE-52 ion exchanger equilibrated with buffer C (0.01 M Tris-HCl, pH 8.4, 1.5 mM EDTA, 10% glycerol (v/v), 1 mM β-mercaptoethanol) were prepared. ^{3}H-dexamethasone-receptor complex was prepared by equilibration of the cytosol in the homogenization buffer with 50 nM labeled hormone at 0°. Then DE-52 minicolumns were loaded with 0.4 ml of this solution. Application of sample was followed by washing with 10 ml buffer C then by elution with a linear KCl gradient. The flow rate was 30 ml h^{-1} and the temperature about 5° throughout the run. Fractions of approximately 0.7 ml were collected. 250 μl from each fraction was transferred directly to the scintillation fluid. KCl concentration was determined by measuring conductivity.

Results

1. *Steroid binding specificity.* In order to determine the specificity of cytosol glucocorticoid receptor in the lymphoid organs of the rat and chicken two concentrations of both natural and synthetic steroids – 9 in all – were used to compete with (^{3}H)-dexamethasone. The competition patterns of rat thymus and spleen were similar (Table 1). Dexamethasone, triamcinolone acetonide and corticoste-

Table 1

Specificity of steroid binding to the glucocorticoid binding protein in rat lymphoid tissues
Competition experiments were performed as described in the Methods. Means of 2—3 independent determinations and S.D.-s are given

Competitor		Specific dexamethasone binding (per cent of control) in cytosol from	
		thymus	spleen
Dexamethasone	10^{-6} M	13.0 ± 2.0	11.7 ± 1.0
	10^{-7} M	36.4 ± 4.0	34.6 ± 3.0
Triamcinolone acetonide	10^{-6} M	11.1 ± 2.6	11.8 ± 0.4
	10^{-7} M	31.2 ± 4.8	30.0 ± 2.7
Corticosterone	10^{-6} M	7.8 ± 0.05	13.2 ± 2.3
	10^{-7} M	29.3 ± 0.55	41.3 ± 0.5
Hydrocortisone	10^{-6} M	12.5 ± 1.9	19.5 ± 6.1
	10^{-7} M	51.7 ± 8.3	64.3 ± 3.7
Cortexolone	10^{-6} M	33.8 ± 0.8	39.4 ± 6.6
	10^{-7} M	84.4 ± 0.9	84.9 ± 3.4
Progesterone	10^{-6} M	18.8 ± 0.55	22.8 ± 4.9
	10^{-7} M	48.3 ± 4.05	54.7 ± 8.4
Aldosterone	10^{-6} M	27.6 ± 0.55	33.4 ± 6.0
	10^{-7} M	76.9 ± 3.2	76.7 ± 4.1
Testosterone	10^{-6} M	109.3 ± 9.3	89.6 ± 0.9
	10^{-7} M	100.4 ± 9.2	96.3 ± 0.4
Estradiol	10^{-6} M	99.8 ± 1.2	95.3 ± 3.1
	10^{-7} M	102.5 ± 9.5	94.3 ± 1.5

rone had the highest affinity for dexamethasone binding sites, hydrocortisone was slightly less able to compete than the other glucocorticoids. Relative affinity of the other steroids was the following: hydrocortisone $\geq$ progesterone $>$ cortexolone $\gg$ $\gg$ estradiol $\approx$ testosterone. Competition patterns of cytosol dexamethasone binding sites prepared from the lymphoid organs of the chicken were also similar (Table 2), save that the affinity of corticosterone for dexamethasone receptor was higher in bursa of Fabricius than in the thymus and spleen. It is to be noted that estradiol and testosterone caused a slight but significant elevation of dexamethasone binding in the spleen. The relative order of competition of the steroids tested for chick dexamethasone receptor was the following: corticosterone $>$ dexamethasone $\approx$ hydrocortisone $>$ cortexolone $\approx$ triamcinolone acetonide $\approx$ progesterone $>$ aldosterone $>$ testosterone $\geq$ estradiol. Triamcinolone acetonide, progesterone and aldosterone had a considerably lower affinity for cytosol dexamethasone binding sites in the chick than in the rat.

2. *Steroid binding kinetics.* To obtain more detailed information about the binding process of dexamethasone to glucocorticoid receptor, association and dis-

Table 2

Specificity of steroid binding to the glucocorticoid binding protein in chicken lymphoid organs
Competition experiments were performed as described in the Methods. Means of two independent determinations and S.D.-s are given

Competitor		Specific dexamethasone binding (per cent of control) in cytosol from		
		thymus	spleen	bursa F.
Dexamethasone	10^{-6} M	9.8 ± 2.0	10.0 ± 2.0	14.4 ± 1.0
	10^{-7} M	29.0 ± 4.0	34.7 ± 5.0	34.9 ± 4.0
Triamcinolone acetonide	10^{-6} M	43.0 ± 1.3	34.0 ± 1.7	38.1 ± 4.1
	10^{-7} M	56.3 ± 3.7	55.7 ± 2.3	44.1 ± 7.1
Corticosterone	10^{-6} M	10.0 ± 2.15	8.9 ± 2.9	7.9 ± 1.1
	10^{-7} M	17.0 ± 5.3	19.4 ± 9.2	6.1 ± 0.8
Hydrocortisone	10^{-6} M	12.2 ± 1.2	14.8 ± 4.3	8.9 ± 0.9
	10^{-7} M	42.5 ± 5.5	47.1 ± 12.2	39.1 ± 4.1
Cortexolone	10^{-6} M	26.4 ± 3.4	28.0 ± 8.2	16.8 ± 4.3
	10^{-7} M	78.8 ± 4.2	62.6 ± 9.0	64.3 ± 14.0
Progesterone	10^{-6} M	30.7 ± 5.3	29.5 ± 7.1	34.3 ± 8.6
	10^{-7} M	63.8 ± 2.7	60.5 ± 7.0	74.3 ± 11.0
Aldosterone	10^{-6} M	60.2 ± 5.9	59.0 ± 8.9	52.1 ± 5.9
	10^{-7} M	105.2 ± 15.0	98.7 ± 21.0	79.8 ± 5.3
Testosterone	10^{-6} M	87.4 ± 8.6	119.9 ± 17.1	79.2 ± 6.2
	10^{-7} M	114.4 ± 10.6	134.5 ± 8.5	97.7 ± 12.4
Estradiol	10^{-6} M	109.7 ± 13.3	125.1 ± 3.1	95.2 ± 12.0
	10^{-7} M	113.5 ± 12.5	131.3 ± 0.8	98.9 ± 9.25

sociation kinetics were followed. Association constants for dexamethasone of different lymphoid organs have been reported elsewhere (Náray et al., 1980). Figure 1 shows the time course of dissociation of the dexamethasone-receptor complex. One representative curve is shown for both species. Dexamethasone-receptor complexes from the different lymphoid organs of a given species dissociated with almost identical kinetics. In the case of the chicken organs, the dissociation curves could be resolved into two phases. The slowly and fast dissociating complexes were present in about the same amounts. The possibility can not be ruled out that the latter is a dexamethasone-transcortin complex. The rate constants of the second phase were comparable to those of rat dissociation curves. These latter fitted straight lines. Rate constants are compiled in Table 3.

Association kinetics of dexamethasone with its cytosol receptors from lymphoid tissues are shown in Fig. 2. Data transformed according to Rodbard (1973) fitted straight lines up to a transformation of about 30% of the receptors to hormone-receptor complex. The association rate constants of chick thymus and bursa receptors for dexamethasone were high (8.1 and 8.5×10^6 M^{-1} at 0°, respectively),

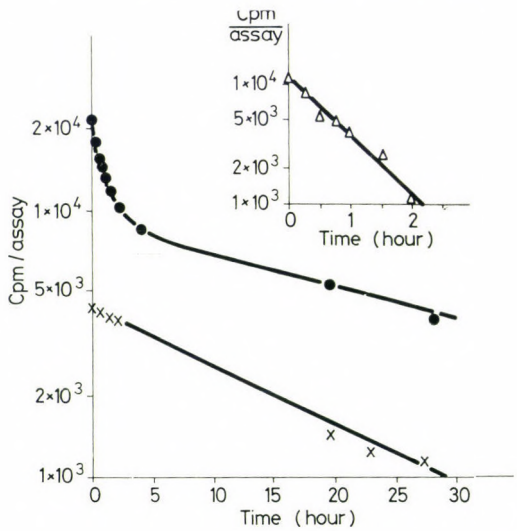

Fig. 1. Dissociation of dexamethasone-receptor complex. Chick (●—●) or rat (×—×) thymus cytosol was allowed to associate with ^{3}H-dexamethasone for 2 hours at 0°, then 5 μM unlabeled dexamethasone was added and the kinetics of displacement was followed. For further details see Materials and methods. The inset shows the first phase of dissociation kinetics (△—△) of chick thymus receptor-hormone complex corrected by subtraction of extrapolated values of the second phase

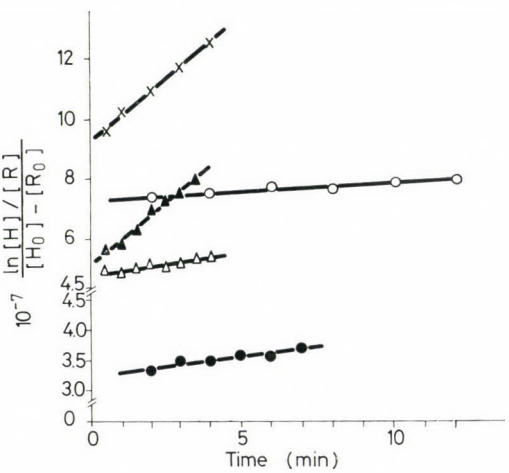

Fig. 2. Association of dexamethasone-receptor complex. Rat thymus (●—●), spleen (○—○), chicken thymus (×—×), spleen (△—△) or bursa of Fabricius (▲—▲) cytosol receptors were mixed with ^{3}H-dexamethasone at 0° at zero time. Final concentrations of ^{3}H-dexamethasone were 9.2×10^{-8} M (rat thymus), 3.1×10^{-8} M (rat spleen) and 2.5×10^{-8} M (chicken tissues). At the times indicated specific dexamethasone binding was determined. Total receptor concentrations were determined from 2.5 hour data by the use of saturation curves. Data are plotted accroding to Rodbard (11), designations: [H], [R] are concentrations of free dexamethasone and receptor, respectively, at time t, $[H_0]$ and $[R_0]$ are initial concentrations of free hormone and receptor. Note the change in ordinate scale

Table 3

Kinetic parameters of hormone-receptor complex formation and dissociation and heat inactivation of dexamethasone receptor

Rate constants were determined from data of Fig. 1. and Fig. 2. and from similar plots where indicated (+). For calculation of K_a rate constants of both the first (*) and second (□) phases of dissociation were used in case of chicken organs. The half-lives of chicken organs cytosol receptors are estimations. After the indicated incubation times (++) about 60% of initial binding was still measurable

Origin of dex receptor	Association rate constant $k_1(M^{-1}\,min^{-1})$	Dissociation rate constant $k_{-1}(min^{-1})$		Equilibrium constant $K_a = \frac{k_1}{k_{-1}}\,(M^{-1})$		Half life of the free receptor (hour)
		1. phase	2. phase	1. phase	2. phase	
Rat thymus	6.5×10^5	8.6×10^{-4}		7.6×10^8		25
spleen	6.1×10^5	$5.7\times10^{-4(+)}$		1.1×10^9		15
Chick thymus	8.1×10^6	1.8×10^{-2}	5.1×10^{-4}	$4.4\times10^{8(*)}$	$1.6\times10^{10(\square)}$	$60^{(++)}$
spleen	1.5×10^6	$1.7\times10^{-2(+)}$	$3.5\times10^{-4(+)}$	$8.6\times10^{7(*)}$	$4.3\times10^{9(\square)}$	$140^{(++)}$
bursa F.	8.5×10^6	$1.7\times10^{-2(+)}$	$4.0\times10^{-4(+)}$	$5.0\times10^{8(*)}$	$2.2\times10^{10(\square)}$	$140^{(++)}$

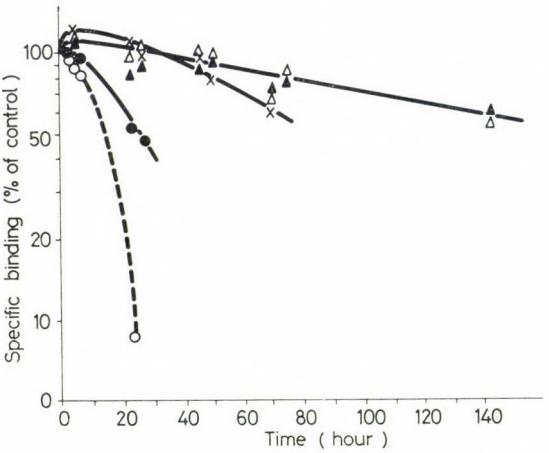

Fig. 3. Heat inactivation of cytosol dexamethasone receptor. Cytosols were prepared in N_2 saturated, DTT-containing buffer (see Materials and methods) and incubated at 0°. Specific dexamethasone binding was determined from aliquots at the times indicated. Per cent of initial binding is plotted vs. time in rat thymus (●— ●), spleen (○— ○), chicken thymus (× — ×), spleen (△— △) and bursa of Fabricius (▲— ▲) cytosols

whereas the other association rate constants were lower by about one order of magnitude (Table 3).

3. *Denaturation of cytosol receptor.* Stability of cytosol dexamethasone receptor was investigated under optimal *in vitro* conditions. Each of the additives EDTA, DTT and glycerol, was found to stabilize the glucocorticoid receptor (Schaumburg, 1972, Schmid et al., 1976). As seen in Fig. 3, glucocorticoid receptors of chicken lymphoid organs were surprisingly stable compared to those of rat tissues. The time course of denaturation did not follow simple, first order kinetics in either case, rather the decay of binding capacity was accelerated in time. A slight initial increase in dexamethasone binding in the cytosol prepared from chicken organs was observed consistently. The half-life values of the receptors are shown in Table 3. As the mechanism of inactivation is not clear it was impossible to assign any rate constant to the time course.

4. *Ion exchange chromatography of dexamethasone-receptor complex.* For ion exchange chromatography of dexamethasone-receptor complexes DE-52 minicolumns were used, equilibrated with 0.01 M Tris-HCl buffer containing 1.5 mM EDTA, 10% glycerol and 1.5 mM β-mercaptoethanol, pH 8.4. To obtain a good separation of the protein-bound ^{3}H-dexamethasone from the free hormone, the columns were washed with four bed volumes of starting buffer before applying the gradient. Dexamethasone in the absence of cytosol did not bind to the column at all (Fig. 4a). Receptor-hormone complexes from all tissues examined were eluted in two peaks at 0.04 – 0.05 M (peak I) and 0.17 – 0.21 M (peak II) KCl concentrations, respectively. Chromatograms of chick bursa of Fabricius and rat spleen

receptor-dexamethasone complexes which are very similar to those of chick and rat thymus receptor-hormone complexes, respectively, are shown in Fig. 4 a–c. The peaks were about equal size in the case of chicken tissues but peak II was much higher than peak I with rat thymus and spleen. These two peaks resisted charcoal treatment, whereas the radioactivity of the peak eluted before the gra-

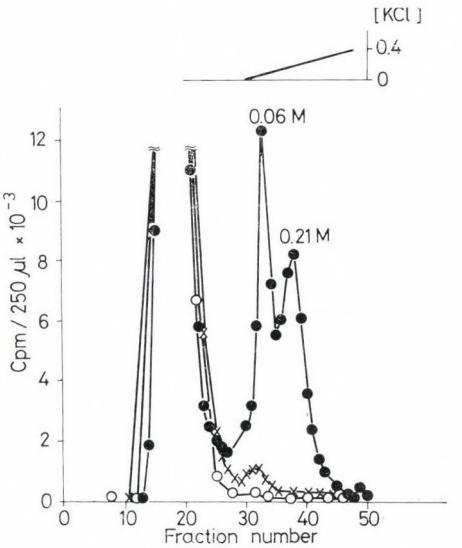

Fig. 4a

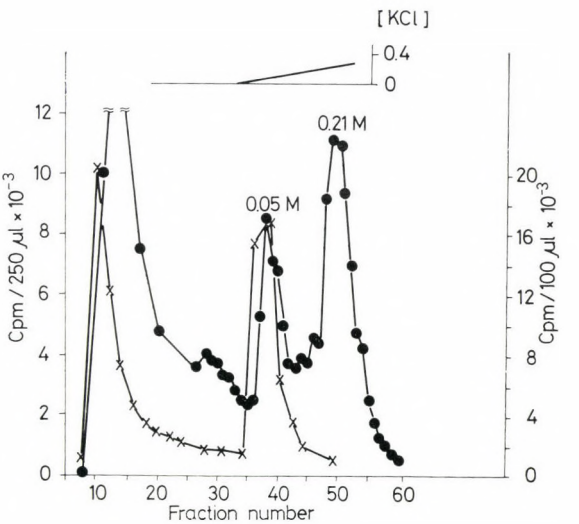

Fig. 4b

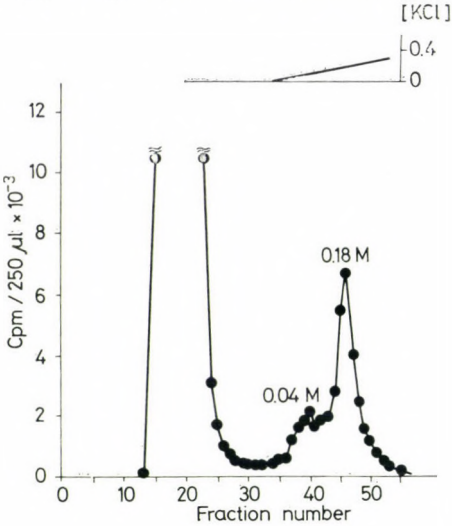

Fig. 4c

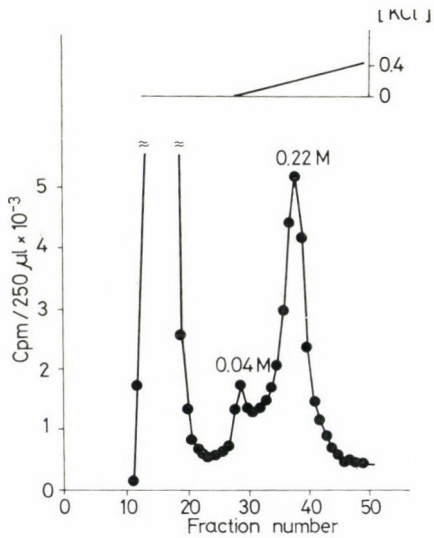

Fig. 4d

Fig. 4. Ion exchange chromatography of dexamethasone-receptor complexes. DE-52 mini-columns were prepared and chromatography was performed as described in Materials and Methods. Panel a: 30 nM ^{3}H-dexamethasone in buffer C (○—○); chick thymus cytosol equilibrated with 30 nM ^{3}H-dexamethasone in the presence (×—×) and in the absence (●—●) of 5 μM unlabeled dexamethasone. Panel b: (●—●), chick spleen cytosol (left ordinate); (×—×), chick serum equilibrated with 25 nM ^{3}H-dexamethasone (right ordinate). Panel c: rat thymus cytosol equilibrated with 25 nM ^{3}H-dexamethasone. Panel d: chick thymus cytosol was equilibrated with 25 nM ^{3}H-dexamethasone. After 2 h of incubation 5 μM of non-radioactive dexamethasone was added to the mixture and it was incubated for two more hours to displace ^{3}H-dexamethasone from the fast dissociating complex, then applied to the column

dient was completely removed by charcoal. The presence of unlabeled dexamethasone in a 100-fold excess during the formation of the hormone-receptor complex eliminated both peaks I and II (Fig. 4a). In preliminary experiments it was determined that no more radioactive peaks appeared up to 0.7 M KCl in any of the tissues. Since chick transcortin, unlike rat transcortin, binds dexamethasone with high affinity (Cochet, Chambaz, 1976) the chick transcortin – ^{3}H-dexamethasone complex was also chromatographed on DE-52 minicolumn. This complex was eluted as a single peak at 0.05 M KCl (Fig. 4b) and thus it might make up part of peak I in the case of chick tissues.

Rat and chick thymus cytosol glucocorticoid receptors could not be separated from each other after mixing, even if the KCl gradient was much shallower than that applied in the above experiments (not shown).

Since on the basis of both kinetic (Fig. 1) and chromatographic (Fig. 4a – b) data dexamethasone-receptor complexes of the chicken tissues appeared to belong to two classes, the following experiments were performed to correlate the results of the two kinds of experiments. ^{3}H-dexamethasone was allowed to associate with chick thymus cytosol receptor for 2 hours at 0°. Labeled hormone was displaced by unlabeled dexamethasone from the fast dissociating complex in a two-hour chase. Then the cytosol was loaded onto a DE-52 minicolumn. As shown by the chromatogram (Fig. 4d), the size of peak I relative to peak II markedly decreased. Thus, we concluded that the fast and slowly dissociating hormone-receptor complex could be separated from each other and they corresponded to the two chromatographic peaks.

Discussion

For a more detailed understanding of the binding process a series of competition and kinetic experiments were performed. On the basis of these studies one can also characterize the glucocorticoid receptors not separated from the cytosol components, which can influence stability and/or binding characteristics (Granberg, Ballard, 1977; DiSorbo et al., 1977; Ishii, Aronow, 1973; Sando et al., 1977). The competition experiments with various steroids revealed – in agreement with earlier studies (Schaumburg, 1972; Rousseau, Schmit 1977) – that only steroid of the pregnane series could displace dexamethasone effectively. Some differences could be observed between chicken and rat lymphoid tissues. The glucocorticoid receptors of chicken organs seemed to be more specific for dexamethasone than those of rat organs (Tables 1, 2). Namely, progesterone, aldosterone and triamcinolone acetonide competed stronger for the rat than for the chicken receptors.

Analysis of the time courses of association and dissociation showed a pronounced difference in the binding processes of chick and rat lymphoid organs. It also brought to light the heterogeneity of cytosol glucocorticoid receptors of the chick which escaped observation when saturation curves were analyzed (Náray et al., 1980). Moreover, association constants obtained from saturation analysis

were much lower than the ratio of association and dissociation rate constants. Similar differences between K_a and k_1/k_{-1} values were previously reported (Schaumburg; 1972, Bell, Munck, 1973; Pratt et al., 1975). These findings were attributed to denaturation of receptors (Bell, Munck, 1973) or monomolecular transformation of the hormone receptor complex (Pratt et al., 1975). In our opinion all these differences are artifacts due to lack of equilibrium under the conditions of saturation experiments (Arányi, 1979).

Differences were found in the inactivation rate at 0° of glucocorticoid receptors from various sources (Lippman, Thompson, 1974; Nielsen et al., 1977). An outstandingly high stability was found in the case of chicken receptors (Table 3). Although the glucocorticoid binding substances examined are clearly discernible by their stability, it should be kept in mind that some cytosol factor(s) not identified as yet may influence stability either positively or negatively (Ishii, Aronow, 1973; Sando et al., 1977; Nielsen et al., 1977).

Ion exchange chromatography seemed to be more effective in the separation of different hormone binding entities than either gel filtration or sucrose gradient centrifugation (Agarwal, Rossier, 1977). Three to four glucocorticoid binding components were detected by DE-52 chromatography in different systems (Schmid et al., 1976; Agarwal, 1977a; Beato, Feigelson, 1972). Distinction could be made between mineralo- and glucocorticoid-receptors or between dexamethasone-receptors of different organs (Agarwal, 1977b). Phosphate gradient appeared to separate activated and non-activated forms of receptor-hormone complexes (Sakaue, Thompson, 1977), while KCl gradient yielded peaks possibly corresponding to receptors with different physiological roles (Grote et al., 1977).

In our system glucocorticoid receptors of each lymphoid tissue could be resolved into two fractions but no difference could be made between binding substances of rat and chicken tissues. The use of minicolumns ensured very short elution times and thus left little chance for the hormone-receptor complex to undergo activation while being chromatographed (Parchman, Litwack, 1977). Since transcortin-dexamethasone complex eluted at 0.05 M KCl, the possibility cannot be ruled out that peak I in chicken cytosols also comprised transcortin, or a transcortin-like intracellular protein (Werthamer et al., 1973).

Generally speaking, kinetic analyses proved to be just as useful as ion exchange chromatography for the characterization of, and distinguishing between glucocorticoid receptors. Equilibrium methods, competition and saturation analyses (see also ref. Náray et al., 1980) appeared to be less informative.

Glucocorticoid receptors from different tissues of rat or chicken showed a similar behaviour by kinetic and chromatographic criteria. Still, hormone-receptor complexes in a given cytosol could be fractionated and, in case of chicken, a two-step dissociation curve was also observed. Receptors from various species with similar binding specificity and affinity displayed very dissimilar stability and their complexes with dexamethasone associated and dissociated differently. The differences observed may not be taken as proper molecular characteristics without further investigations, because possible cytosol factors (see above) may greatly

influence the parameters studied. Nevertheless, the characteristics of hormone binding to its receptor can be physiologically relevant only in the cytosol microenvironment.

On the basis of these and previous results, sensitivity differences between various species and tissues shown elsewhere (Náray et al., 1980) could not be explained by either variations of receptor content or physicochemical characteristics of receptors and/or binding processes. Thus, we conclude that differences in sensitivity are most probably due to some yet unidentified process(es) or factor(s) that act beyond nuclear transfer or concomitantly with hormone-receptor binding and activation. This conclusion is in accord with the results on lymphocytes of leukemic patients (Duval, Homo, 1979).

The authors are highly indebted to Drs I. Horváth and E. B. Thompson for instructive discussion and criticisms. Thanks are due to Miss Márta Mezei for skilled technical assistance. This work was partially supported by G. Richter Ltd. Hungary.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 199–203 (1980)

Video-Fluorimetric Evaluation of Human Blood Serum Electrophoretograms

(Short Communication)

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(Received October 5, 1979)

The relative concentration of serum protein fractions to total serum protein, and changes in these quantities are of great clinical and pathological importance. The considerable difficulties of screening large numbers of samples in routine laboratory work called for the elaboration of several methods suitable for the separation of various serum proteins. The most frequently used methods involve the staining of the electrophoretograms by one of the commonly used protein dyes (e.g. Ponceau red, amido black) and subsequent photometric or densitometric measurement of the resulting colored spots. Characteristic errors (standard deviation as percentage of the mean) of 5.0%, 17.2%, 11.1%, 12.7% and 16.3% have been determined for the relative amounts of the different protein fractions albumin, α_1, α_2, β and γ globulins, respectively, by evaluating Ponceau S stained human blood serum electrophoretograms on cellulose acetate support (Schmidtmann et al., 1969). The concentration values determined depend very much on the actual method of producing and evaluating the electrophoretograms.

A fast and more accurate method has been established for staining cellulose acetate and agar gel electrophoretograms with a fluorescent primary amine reagent. It has also been used for the detection of peptides on thin-layer chromatograms (Udenfriend et al., 1972a, b). Our main aim was to evaluate quantitatively such protein electrophoretograms by video-fluorimetric detection of their fluorescence. Both staining with the applied fluorescent dye, fluorescamine (4-phenylspiro furan-2(3H),1'-phtalan-3,3-dione) and video-fluorimetric evaluation of the electrophoretograms can be performed very fast and accurately compared to the conventional methods.

The serum protein electrophoretograms on cellulose acetate strips were prepared according to the usual procedure by using 50 mM borate buffer, pH 8.5, and less than 10 μl aliquots.

The developed electrophoretograms were dried in air at room temperature and stained with fluorescamine according to the following procedure:

The cellulose acetate strips were placed in a plastic trough and pretreated with 50 mM borate buffer, pH 8.5, required as an aqueous medium for the chemical reaction to occur between primary amines and the dye.

After 1–2 minutes the bulk of the buffer was removed except a very thin layer, which was left to cover the electrophoretograms. The wet electrophoretograms were then uniformly sprayed with 3–4 ml of fluorescamine solution (0.1% w/v in dioxane). Solutions of fluorescamine were freshly prepared each day and stored at room temperature in a stoppered tube. After 30–60 seconds the samples were washed 2–3 times with borate buffer and dried in air.

The serum protein electrophoretograms on agar gel media were also prepared according to the usual procedure by using 2–4 μl serum samples, veronal-sodium acetate buffer, pH 8.6, and 8 mA/sheet for approximately 2 hours. The developed electrophoretograms were fixed in 10% acetic acid and subsequently stained according to the method described above for the staining of cellulose acetate electrophoretograms or with amido black.

Following the above procedures the samples are ready for fluorescence intensity measurements. According to our experience the dried electrophoretograms were stable and could be stored in the dark for a few days without loss of fluorescence intensity.

Figure 1 shows fluorescamine and amido black stained human blood serum electrophoretograms containing equal amounts of serum of the same origin on agar gel support for visual comparison (Fig. 1).

The quantitative evaluation of the electrophoretograms was carried out by the use of modified TELECHROM OE-976 type video-densitometer (Eurolab, Munich GFR). The modification of the video-densitometer, to perform video-fluorimetric measurements, required an appropriate sample holder and excitation unit to include the Sylvana F8T5/BLB fluorescent tubes (366 nm) used as the exciting light sources. Since the emission spectrum of the protein-fluorescamine complexes displayed a significant fluorescence well beyond the 485 nm maximum, an ORWO cut-off filter (485 nm) was applied in front of the sensing optics of the instrument to increase the sensitivity of the measurements. Moreover, the electronics of the TELECHROM video-densitometer was slightly modified by attaching to it a so-called positive-negative switch to transform the white spots of the fluorescent electrophoretograms into measurable dark spots on the screen. This was necessary because the electronics of the instrument was originally developed for conventional densitometric applications.

The fluorescence intensity measurements for the characterization of the different serum protein fractions on the electrophoretograms were performed in a very convenient way according to the procedure described earlier for video-densitometric evaluation of TLC chromatograms (Dévényi, 1976). The fluorescent electrophoretograms were uniformly illuminated with UV light and their negative images were displayed on the monitor of the TELECHROM instrument for proper alignment. Rectangular areas ("windows") were set and scanned and the Vidicon-tube of the TELECHROM instrument was used as the detector of the system. The signals were electronically integrated to gain fluorescence intensity data.

The base line of the instrument was set to those portions of the stained blood serum electrophoretograms which contained no protein. Figure 2 represents the

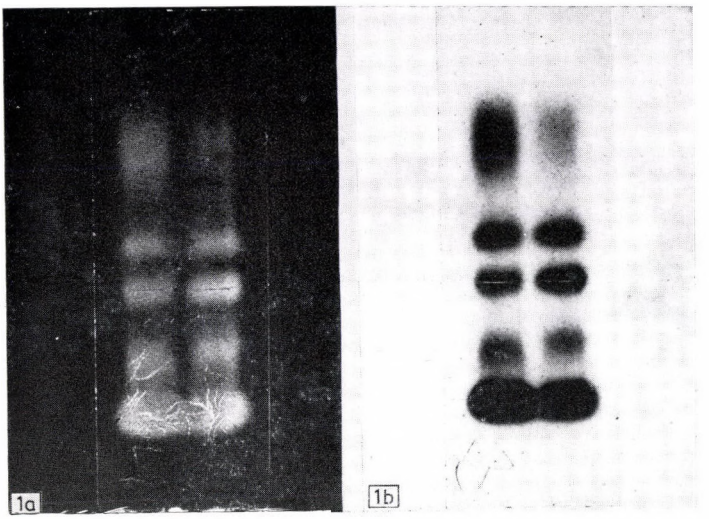

Fig. 1. Fluorescence characteristics of fluorescamine-stained human blood serum. Agar electrophoretogram stained with fluorescamine, photographed under ultraviolet illumination (a). Blood serum electrophoretogram of the same origin stained with amido black (b)

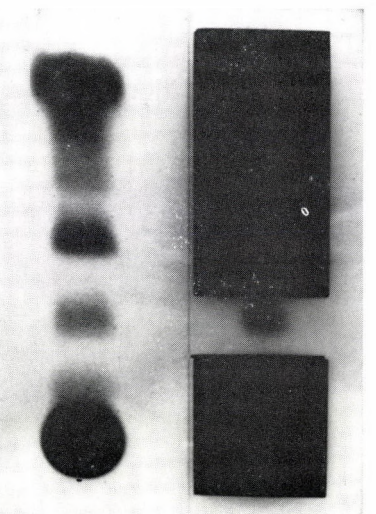

Fig. 2. Measurement of the fluorescence intensity of blood serum proteins on cellulose acetate support as displayed by the TELECHROM video-fluorimeter. The white window within the black one is for the determination of a single, individual component as percentage of the total protein in the sample (see the percentage values in Table 1). The perpendicular straight line across the screen is the base line of the instrument used as reference for electronic scanning of the "window"

measurement of fluorescence intensity of blood serum proteins on cellulose acetate support by TELECHROM. (For details see the legends to Fig. 2.)

During the elaboration of the above staining and detection method of fluorescamine-labeled cellulose acetate membrane and agar gel electrophoretograms the following advantages of the applied dye were exploited (Weigele, 1972): No fluorescence from the dye is observed when it is dissolved in an organic solvent. Mixing the dye in excess with primary-amine containing proteins in aqueous environment results in the formation of a covalent complex having strong fluorescence with excitation and emission maxima at 385 and 485 nm, respectively. The chemical reaction is very rapid and takes place within a few seconds. The unreacted dye is decomposed in the aqueous environment into nonfluorescent products. The well separated excitation and emission maxima of the dye make the fluorescence of the dye-protein complex easily detectable.

Experiments were done to study the linearity of the fluorescent staining and video-fluorimetric detection method at protein concentrations corresponding to those used in routine serum electrophoresis. As shown in Fig. 3 a linear correlation was found between fluorescence intensity and protein concentration within the applied concentration range. The lowest well measurable quantity of the applied HSA (human serum albumin) was 100 pmoles.

Fluorescence intensity measurements were carried out simultaneously with the above measurements by a thin-layer chromatographic (TLC) apparatus attached to a Hitachi MPF-4 spectrophotofluorimeter to control the results obtained by video-fluorimetry. The total fluorescence intensity corresponding to specific protein fractions in the electrophoretograms was estimated by planimetry. The video-fluorimetric results were in good agreement with those obtained by the TLC spectrophotofluorimetric method.

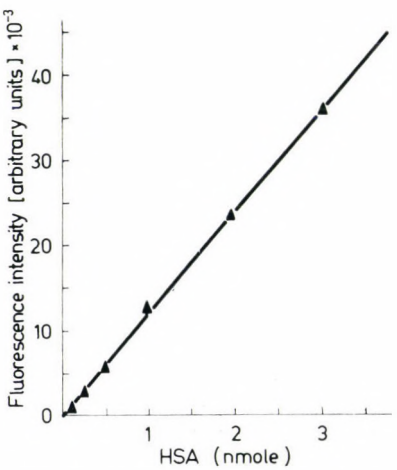

Fig. 3. Variation of the fluorescence intensity of fluorescamine-stained human serum albumin with protein concentration

Table 1

Fluorescence of human blood serum fractions on cellulose acetate support

(Means of 5 measurements are given in the table)

Protein fraction	Area measured by TLC apparatus % (arbitrary units)		Relative error %	Fluorescence intensity measured by TELECHROME (arbitrary units)	%	Relative error %
Albumin	64.0	61.7	1.5	36 295	59.3	1.9
α_1 globulin	3.2	3.1	10.1	2 575	4.2	11.5
α_2 globulin	10.8	10.4	8.4	5 814	9.5	9.6
β globulin	9.4	9.1	5.0	5 835	9.5	7.4
γ globulin	16.3	15.7	11.4	10 696	17.5	13.6
Total	103.7	100.0		61 215	100.0	

Table 1 summarizes the results obtained by evaluating a representative human blood serum electrophoretogram by both methods.

The aim of the present study was to develop a very fast and reliable staining method for the evaluation of blood serum electrophoretograms. The fluorescent dye applied, fluorescamine, was found superior to most of the conventional protein dyes. The satining of the developed electrophoretograms is very fast and no fixation procedure is required. The linearity of the staining and detection method within the tested protein concentration range has been proven. Video-fluorimetric evaluation of the fluorescamine-stained electrophoretograms was established. The video-fluorimeter provides data in a very convenient, digital form and is capable of evaluating large numbers of chromatograms with high speed. Moreover, the evaluation of the results can be automatized by transferring the data from the instrument into an online connected minicomputer.

Thanks are due to Dr T. Dévényi for his constant interest and helpful discussion throughout the work.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 205–209 (1980)

The Action of Non-ionic Detergents on the Extraction of Phospholipids and Proteins from Rat Liver and Rabbit Heart Membranes

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(Received September 27, 1979)

Triton X-100 and Lubrol PX at similar concentrations extracted more phospholipid than protein from rat liver and rabbit heart membranes, as compared with their original protein and phospholipid contents, respectively. All the types of membrane phospholipid were solubilized from rat liver membrane with the used non ionic detergents, but less sphingomyelin was extracted than phosphatidylcholine and phosphatidylethanolamine.

Introduction

Non-ionic detergents induce changes in the properties of biological membranes by solubilizing them into lipoproteins and mixed micelles (Bont et al., 1969; Helenius, Söderlund, 1973). Evidence indicates that non-ionic detergents extract proteins from membranes in most cases without loss of biological activity, therefore they are useful for the separation of receptor proteins (Cuatrecasas, 1972), antigens and membrane enzymes (Lisman et al., 1971; Suginaki et al., 1972). By this technique it is possible to investigate the role of lipids in the activity of membrane bound enzymes (i.e. adenylate cyclase). However, the mechanism of the action of detergents on the membranes is still not known in detail.

The purpose of the present work was to study the extraction of phospholipids and proteins by several nonionic detergents and to investigate the relationship between solubilized phospholipids and proteins from rat liver and rabbit heart membrane.

Materials and methods

Non-ionic detergents, Triton X-100, Triton X-305 and Lubrol PX were obtained from Sigma Chemical Co, St. Louis, Mo. U.S.A. Female PVG/c rats (6–8 weeks old) and New Zealand Albino rabbits of both sexes were used.

Abbrevations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

Preparation of heart plasma membranes. Rabbit heart membrane was prepared according to the method of Louis and Sulakhe (1976) omitting the isolation of membrane fractions by sucrose gradient ultracentrifugation.

Preparation of crude liver membranes. Rat liver membrane was isolated using the method of Neville (1968) up to step 11.

Solubilization of membranes with non-ionic detergents. Membrane samples were treated with non-ionic detergent in 20 mM Tris-HCl, pH 7.4. Appropriate volumes of water and detergent (5% w/v) were added to the membrane suspensions to give the required concentration of detergent and a final concentration of 3 mg/ml membrane protein. Detergent-membrane mixture were incubated for 30 min at 4 °C and shaken by hand every 5 min. The solubilized fractions were obtained by the ultracentrifugation method. The solubilized fraction was centrifugated at 105 000*xg* in a Spinco ultracentrifuge (Rotor SW_{39}) for 60 min at 4 °C and the supernatant was used for the analyses.

Analytical methods. Extraction of phospholipid and purification of chloroform phase was done according to Folch et al. (1957). Phospholipid phosphorus was quantitatively measured by the method of Kahovcová et al. (1969). The proteins were determined by the method of Lowry et al. (1951) with the modification for proteolipids as described by Dulley and Grieve (1975). Phospholipids were separated by thin-layer chromatography according to Neskovic and Rostic (1968) and the phosphate concentration was estimated after digestion with 50% H_2SO_4 at 180 °C for 1 hour (Turner, 1970).

Results

The capability of non-ionic detergents of extracting phospholipids and proteins from rat liver membrane is shown in Table 1. Solubilization of proteins proved to depend on the concentration of the detergent. However, there were significant differences in solubilizing power among the detergents investigated. Triton X-305 extracted less membrane protein than Lubrol PX and Triton X-100 at all detergent concentrations investigated. When the ratio of Triton X-100 to membrane protein (wt/wt) was 2 : 3, about 40% of the total membrane protein content was solubilized, and when this ratio was increased to 3.3 : 1 the extent of solubilization could be elevated to about 50% of the total membrane protein content. On the contrary, Triton X-305 extracted only about 25% of the total membrane protein when the ratio of the detergent to membrane protein was 3.3 : 1. The order of potency to solubilize membrane phospholipids was the same as that observed for proteins and was also concentration dependent. The results obtained with the extraction of phospolipids and proteins from rabbit heart membrane were the same as those from rat liver membrane (Table 1), but the extent of solubilization was less for both phospholipids and proteins. Even when the detergent concentration was increased to 2.5% Triton X-100 did not extract more than 40% protein from rabbit heart membrane.

Table 1

Solubilization of phospholipids and proteins from rat liver and rabbit heart membrane at different detergent concentrations

Detergent concentration (%)		Rat liver membrane		Rabbit heart membrane	
		Protein	Phospholipid	Protein	Phospholipid
		(mg/ml)			
Control		3.00 ± 0.40	1.82 ± 0.17	3.00 ± 0.36	1.59 ± 0.18
Triton X-100	0.2	0.96 ± 0.09	0.75 ± 0.08	0.60 ± 0.07	0.31 ± 0.03
	0.5	1.38 ± 0.12	1.04 ± 0.09	0.85 ± 0.09	0.52 ± 0.04
	1.0	1.65 ± 0.16	1.53 ± 0.14	1.03 ± 0.08	0.76 ± 0.08
	2.5	—	—	1.17 ± 0.10	0.96 ± 0.10
Lubrol PX	0.2	0.88 ± 0.08	0.68 ± 0.06	0.52 ± 0.06	0.26 ± 0.03
	0.5	1.15 ± 0.01	0.91 ± 0.09	0.66 ± 0.07	0.32 ± 0.04
	1.0	1.51 ± 0.14	1.33 ± 0.14	0.76 ± 0.09	0.41 ± 0.05
	2.5	—	—	0.84 ± 0.10	0.50 ± 0.05
Triton X-305	0.2	0.37 ± 0.06	0.15 ± 0.02	0.33 ± 0.03	0.04 ± 0.01
	0.5	0.56 ± 0.07	0.23 ± 0.03	0.41 ± 0.04	0.08 ± 0.01
	1.0	0.62 ± 0.09	0.33 ± 0.04	0.49 ± 0.06	0.09 ± 0.02
	2.5	—	—	0.55 ± 0.06	0.10 ± 0.02

The data are the means of 3 expts. ± S.D.

Table 2

Phospholipid conposition of rat liver membranes and membrane fractions solubilized with non-ionic detergents

The results are expressed as percentages of total phospholipid phosphorus recovered from the thin-layer chromatography plates

The data are the means of 3 expts. ± S.D.

Preparations	PE	PC	SM	PI	PS
Rat liver membrane	25.19 ± 1.49	45.1 ± 2.1	14.97 ± 2.1	6.93 ± 0.4	8.56 ± 0.3
Membrane fraction solubilized with the non-ionic detergents					
Triton X-100 1%	25.43 ± 1.3	52.9 ± 2.3	5.86 ± 0.6	8.76 ± 0.21	7.00 ± 0.3
Lubrol PX 1%	25.15 ± 0.9	50.78 ± 1.9	6.06 ± 0.92	8.19 ± 0.91	7.75 ± 0.2
Triton X-305 1%	26.44 ± 1.4	49.58 ± 1.6	6.61 ± 0.7	9.09 ± 0.2	8.46 ± 0.2

Table 3

Phospholipid content of rabbit heart membrane fractions obtained by solubilization with different non-ionic detergents

The figures represent μg phospholipid per mg protein

The values are means of three separate experiments $\pm$ S.D.

Non-ionic detergent concentration %	Intact rabbit heart membrane	Rabbit heart membrane treated with		
		Triton X-100	Lubrol PX	Triton X-305
–	22.0 $\pm$ 2.5	–	–	–
0.2	–	14.0 $\pm$ 1.6	8.4 $\pm$ 0.7	4.9 $\pm$ 0.4
0.5	–	22.0 $\pm$ 2.7	17.4 $\pm$ 1.6	6.4 $\pm$ 0.5
1.0	–	29.0 $\pm$ 3.1	19.5 $\pm$ 2.1	6.6 $\pm$ 0.6
2.5	–	36.0 $\pm$ 3.8	23.2 $\pm$ 2.5	6.6 $\pm$ 0.5

At detergent concentrations from 0.5 to 1%, the composition of the extracted phospholipids did not differ qualitatively. When the detergent concentration was lowered to 0.2%, sphingomyelin was less solubilized. As shown in Table 2, non-ionic detergents had less capability of solubilizing sphingomyelin than other phospholipids. The ratio of solubilized phospholipids to solubilized protein depended on the detergent concentration (Table 3). With Triton X-100 and Lubrol PX, the ratio of phospholipid to protein increased with increasing detergent concentrations. In contrast, the ratio of phospholipid to solubilized membrane did not change in the range of 0.5 to 2.5% Triton X-305.

Discussion

The term hydrophile – lipophile balance (H. L. B.) has been introduced to characterize the size and strength of opposing hydrophilic and hydrophobic groups in non-ionic surfactants. The striking correlation between detergent solubilization of membrane enzymes and detergent H.L.B. number has been reported from several laboratories (Umbreit, Skomenger, 1972, 1973; Storm et al., 1976). It has been shown that non-ionic detergents with H.L.B. numbers in the range from 12.5 to 14.5 could extract not only relatively much more membrane phospholipids but also much more membrane proteins than those with a H.L.B. number in the range of about 17, such as Triton X-305. Tzagoloff and Penefsky (1971) found that Triton X-100 and other non-ionic detergents (mostly polyoxyethylene alcohols like Lubrol) can solubilize a number of integral membrane proteins without loss of their biological activities. Our results suggest that Triton X-100 and Lubrol PX may act as "wedges", which destroy the natural orientation of the lipid bilayer of the membrane. If the detergent concentration is increased not only the bulk lipids in the bilayer but also the boundary lipids can be exchanged for detergent and trans-

ferred into mixed micelles of lipid and detergent, resulting in an almost complete separation of lipid from proteolipids. Over 50% of the total membrane protein was extracted and 80% of the total membrane phospholipid was solubilized. Our results also show that membrane lipids can be selectively extracted by non-ionic detergents: they solubilize more phosphatidylcholine than other phospholipids and less sphingomyelin. Solvent extraction also results in a selective removal of phospholipids from membranes (Réthy et al., 1971) but in this case, unlike extraction by non-ionic detergents, the relative amount of sphingomyelin is larger. The major difference between the two treatments is that solvent extraction affects probably only the outer layer of membranes, while the detergents attack the whole structure. Since sphingomyelin is a constituent of the outer layer of plasma membranes (Emmelot, Hoeven, 1975), it is probable that membrane domains rich in these phospholipids can not interact with the detergent molecules. An indirect support for this assumption is that 55–60% of the total membrane cholesterol remained unsolubilized using Lubrol PX (data not shown). Cholesterol and sphingomyelin have been shown to be associated in biological membranes (Demel et al., 1977). The observed selectivity of lipid removal from membranes may result from specific interactions between lipids and other membrane components (mainly protein), which would influence their extractability.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 211—215 (1980)

A New Approach to Studying Cell-Cell Communication by the Use of Flow Fluorimetry

(Short Communication)

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(Received August 15, 1979)

Communication between animal cells can take place either by direct physical contact or through interactions by diffusion of molecular messages from one cell to the other. Presumably water-soluble molecules function in the second mechanism, while lipophilic molecules contribute to the first group of interactions.

Collard et al. (1978) have described a model system for studying direct cell suspensions using the lipophilic fluorescent probe DPH. Translocation of the probe from labelled cells to unlabelled ones was monitored by following the change in the fluorescence of the labelled and initially unlabelled suspensions subsequent to their mixing. Control experiments were conducted to detect passage of the probe through the water phase and it was concluded that contact of colliding cells was the way of DPH transfer. Exchange of radioactive-labelled lipids between artificial vesicles and cells has also been reported and the contact mechanism considered by Pagano and Huang (1975).

DPH has been widely used for the examination of membrane fluidity in intact cells, isolated membranes and their constituents (Obrenovitch et al., 1978; Gilmore et al., 1979a, b; Van Hoeven et al., 1979) with fluorescence techniques including flow fluorimetry (Arndt-Jovin, Jovin, 1976; Jovin, 1977), first introduced as a lipid probe by Shinitzky and co-workers (Shinitzky et al., 1971; Shinitzky, Barenholz, 1974; Shinitzky, Inbar, 1974). In this study flow fluorimetric analysis was applied to follow the distribution of DPH among suspended cells on a single-cell basis, measuring the fluorescence of each cell individually.

Human peripheral blood lymphocytes were prepared on Ficoll-Isopaque gradients. The cells were washed three times with PBS prior to labelling, to avoid possible lipid-containing debris. For both labelling and flow fluorimetric analyses 2×10^6 cells per ml were used. Human embryonic fibroblast secondary cultures grown as monolayers in minimal essential medium supplemented with 10% fetal calf serum and antibiotics, were used for flow fluorimetric studies after suspending them by treatment with a 0.02% trypsin solution. DPH (Sigma) was dissolved at 4×10^{-3} M concentration in tetrahydrofuran, then diluted 400 times with PBS and

Abbreviations: DPH, 1,6-diphenyl-1,3,5 hexatriene; PBS, phosphate buffered saline (pH 7.4).

stirred for one hour at room temperature. Cells in PBS were added to make a final DPH concentration of 5×10^{-6} M and incubated for labelling at room temperature for 30 minutes. After staining with DPH the cells were centrifuged and washed four times with PBS. Because of rapid fading of DPH, the dye was protected from light throughout the experiment. The flow fluorimetric measurements were carried out in a Becton-Dickinson Fluorescence Activated Cell Sorter (FACS III). In this system the cell suspension stored in the sample tube passes through a plastic tubing to a nozzle which is mechanically coupled to a piezoelectric transducer that breaks the fluid stream into small droplets, each containing a single cell or none. The cells are illuminated by an argon-ion laser, and the scattered light and the fluorescence detected by photomultiplier tubes. The intensities (both scatter and fluorescence) are stored in a multichannel pulse height analyzer and displayed as frequency distribution histograms. The 360 nm band of the laser was set for excitation and a 480 nm 03 FIV 017, Melles-Griot, interference filter was used on the emission side. The laser light intensity was highly stabilized during the experiments.

Figure 1 shows the distribution of fluorescence of cells 2, 7 and 30 minutes after mixing the labelled population (D) of human peripheral blood lymphocytes with the unstained control lymphocytes (C). These cells were incubated together in the sample tube of the instrument and aliquots were flown through at the times indicated. The control was passed through the instrument after the labelled cells, in

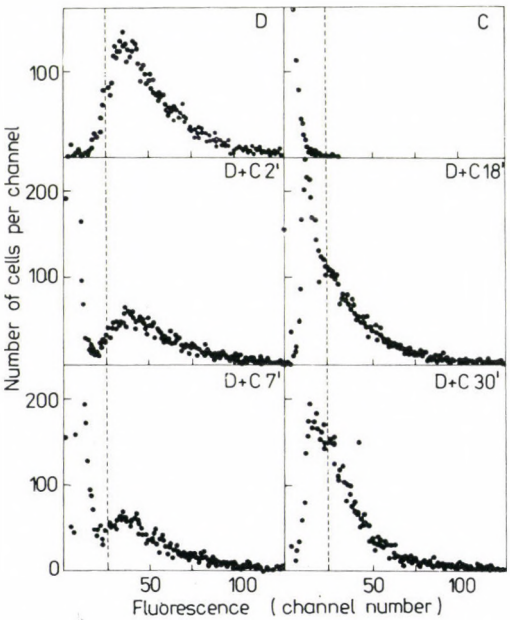

Fig. 1. Frequency distribution of fluorescence of DPH-labelled (D), unlabelled (C) human peripheral blood lymphocytes and that of a 1 : 1 mixture of D and C samples, recorded at 2, 7, 18 and 30 minutes after mixing

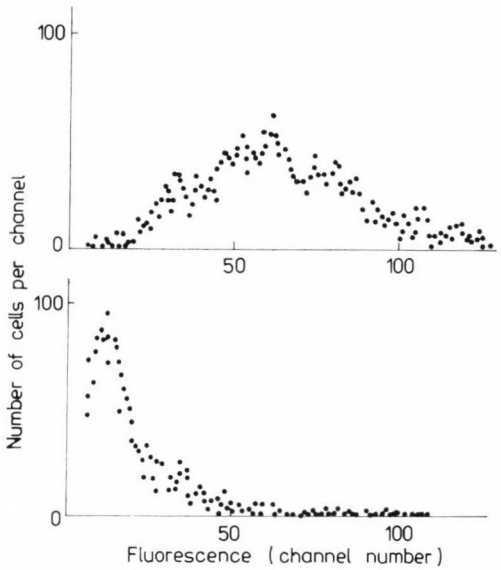

Fig. 2. Frequency distribution of fluorescence of DPH-labelled, nonwashed human embryonic fibroblasts (top) and that of a mixture of one volume of labelled with two volumes of unlabelled cells, recorded at 2 minutes after mixing (bottom)

order to estimate the extent to which the plastic tubing in which the cells flew mediated the dye on to the unlabelled population. It is apparent that the cells acquired a small intensity of fluorescence in this way. Soon after the addition of an equal volume of C to D, the number of cells showing average fluorescence decreased by a factor of two. The average fluorescence of the cell population, however, was unchanged, revealing that in the mixture every second cell belonged to the D population. Later the peaks gradually shifted toward lower intensity values indicating redistribution of the dye among the cells. The little fluorescence contamination passed on to the cells by the tubing was undetectable at 30 minutes, since all the cells contained more DPH by then. The peak of C and that of D almost fused at 30 minutes to one common distribution of fluorescence, approaching a new balance.

When washing of cells after labelling was omitted from the procedure, the new balance was established in a few minutes as shown in Fig. 2 (for human embryonic fibroblasts). In one experiment a similar quick redistribution of DPH was found, though washing of cells (lymphocytes) was not excluded from the procedure (data not shown). To interpret this finding we assume that tetrahydrofuran dispersion or accidental cell-derived lipid debris may enhance dye translocation by playing a mediating role.

Since DPH is practically insoluble in water, dye translocation is probably due to direct transfer of DPH molecules during the encounter time of the two kinds

of cells. A slow "leaking" through the water phase, however, cannot be ruled out as a possible mechanism. (We failed to detect the translocation process by fluorescence microscopy, because of the rapid fading of DPH.)

The control experiments of Collard et al. (1978) may not give unequivocal evidence either. They applied a Ficoll-Isopaque layer to separate the DPH labelled monolayer of cells from a suspension of unlabelled ones in the same flask. Only a small degree of translabelling through the layer, of the suspended cells was found, in contrary with the result of the control experiment in which the suspended cells were mixed with the same amount of Ficoll-Isopaque that was used for the separating layer. However, the Ficoll-Isopaque layer is not identical for certain, in its DPH "permeability" to the Ficoll-Isopaque dispersion that served as control. Thus it cannot be excluded that DPH translocation from the monolayer to the suspension was prevented by a hindered diffusion through the Ficoll-Isopaque layer and not because no contact between the cells was possible. Attempts are in progress in our laboratory to clarify the mode of DPH exchange in this type of experiments. It is interesting to note that the state of lymphocytes flowing through a tubing during fluorimetric analysis resembles, to a certain degree, cells within the bloodstream. The fact that the plastic tubing transferred DPH between the C and D populations may suggest that the wall of the blood vessels can act in a similar way.

With the present study we confirmed previous findings on DPH translocation with a single-cell screening technique. However, we emphasize the need for a better understanding of the underlying mechanism. We consider the described system as a new, versatile method applicable for investigations concerning cell communication.

We hope that further investigations on this system will prove useful in learning how cells exchange a mobile, lipophilic molecule like DPH, so that a similar possibility can be evaluated for other lipophilic molecules of the functioning animal cell.

Thanks are due to Prof. S. Damjanovich for discussions and critical reading of the manuscript, to Dr L. Gergely for providing human embryonic fibroblast cultures and to Mrs. M. Nagy for preparing human peripheral blood lymphocytes used in the experiments.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 217—222 (1980)

A Simple Procedure for the Preparation of Electrophoretically Homogeneous α-actinin from Rabbit Muscle

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(Received June 8, 1979)

A simplified procedure for the preparation of α-actinin from rabbit skeletal muscle is described. Use is made of the observation that actin is completely eliminated by denaturation, when myofibrils are dissolved in 0.6 M $MgCl_2$ and thereafter $MgCl_2$ is removed by dialysis.

Introduction

α-actinin was first described by Ebashi and Ebashi (1965) and was later identified as the main protein component of the Z-membrane of striated muscle (Masaki et al., 1967). The recent finding that α-actinin is localized in the actomyosin-type cytoskeletal complex (Schollmeyer et al., 1976; Jockush et al., 1977) adds considerably to the interest in this protein.

All procedures proposed so far for obtaining α-actinin liberate this protein from the myofibrillar complex by prolonged extraction with a very low ionic strength salt solution (e.g. 1 mM Tris-HCl buffer, pH 8.5). This results in the production of a rather dilute crude extract which contains practically all the α-actinin (Suzuki et al., 1976). To obtain a homogeneous preparation, this extract must be subjected to ammonium sulfate fractionation, two cycles of chromatography on DEAE-cellulose columns and one on hydroxyapatite (Suzuki et al., 1976). We describe here an alternative, more simple procedure.

Materials and methods

Myosin was obtained from the Hasselbach-Schneider extract of myofibrils by two reprecipitations by dilution to an ionic strength 0.35 μ. A final clarification by centrifugation at 50 000 *g* for 90 min was applied.

Actin was extracted at 0 °C by 0.2 mM ATP from a dried muscle powder prepared according to Bárány et al. (1954) and purified by one polymerization-depolymerization cycle according to Mommaerts (1951).

Actomyosin was a 1 : 2 mixture of actin and myosin.

ATP-ase activity was measured according to Arakawa et al. (1970) at 25 °C in 2 ml samples. Phosphate liberated was measured according to Fiske and Subba-Row (1925) with inclusion of 0.4% SDS (cf. Tashima, 1975).

Turbidity measurements were carried out according to Arakawa et al. (1970).

SDS polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (1969) in 6% polyacrylamide gels.

*Analytical ultracentrifugation** of samples at a concentration of 1 mg/ml was carried out in a MOM 3170 analytical ultracentrifuge.

Myosin concentration was measured by the biuret method (Gornall et al., 1949) and *actin* according to Houk and Ue (1974). The concentration of pure *α-actinin* was measured by extinction at 280 nm. An $E_{280}^{1\%} = 13.5$** was used, based on nitrogen determinations according to Jaenicke (1974). The nitrogen content of α-actinin was calculated on the basis of amino acid analyses described by Robson et al. (1970).

Results and discussion

The elaboration of the more simple procedure proposed here came from the observation that actin, the most persistent contamination of crude α-actining preparations can be completely eliminated by denaturation with 0.6 M $MgCl_2$ (Biró, Venyaminov, 1979).

When myofibrils are suspended in 0.6 M $MgCl_2$ they dissolve virtually completely. On eliminating $MgCl_2$ by dialysis, the bulk of proteins precipitates and we obtain a supernatant which contains all the α-actinin, without any trace of actin. As documented below, α-actinin retains its full biochemical activity during this procedure. Pure α-actinin contaminated only by some M-protein could be obtained from this crude extract by one ammonium sulfate fractionation step followed by a single chromatography on DEAE-cellulose. To avoid contamination by M-protein myofibrils were extracted, prior to $MgCl_2$ treatment by Hasselbach-Schneider's solution which extracts in addition to a considerable part of myosin all M-protein (Masaki, Takaiti, 1974). This goes with the loss of some α-actinin but it is essential for avoiding contamination by M-protein.

The detailed procedure for the preparation of crude extract was the following. The myofibril mass obtained from 100 g of rabbit white muscles by a slightly modified version of the procedure of Perry and Corsi (1958) was extracted for 90 min with 300 ml Hasselbach and Schneider's solution (0.6 M KCl, 0.1 M K-phosphate buffer, 10 mM potassium pyrophosphate, 1 mM $MgCl_2$, pH 6.4). The precipitate was washed six times, once with 300 ml of the above solution, five times with 500 ml each time of 0.02 M borate-borax buffer, pH 7.4, 0.02 M KCl. All these centrifugations were done at about 3000 *g*.

The resulting precipitate was thoroughly triturated in a mortar with an amount of concentrated $MgCl_2$ solution to give in the final paste a concentration of

* Thanks are due to Dr Zs. Lakatos, Enzymology Department, Biological Research Center, Hungarian Academy of Sciences, for the ultracentrifugation experiments.

** The measurements giving this extinction coefficient were kindly performed by Dr. S. Yu. Venyaminov, Protein Research Institute, Soviet Academy of Sciences, Poustchino, URSS.

Table 1

Influence of α-actinin on the Mg-ATP-ase activity of "synthetic" actomyosin

Conditions of measurement: 50 μg/ml actomyosin, 30 mM Tris-acetate, pH 7; 1 mM $MgCl_2$ 0.05 mM $CaCl_2$; 1 mM ATP and α-actinin added to 2 ml samples as shown in the Table. Incubation for 3 min at 25 °C

α-actinin added μg	ATP-ase activity $\frac{\mu molP_i}{mg\ actomyosin \cdot min}$
—	0.293
6.5	0.345
13	0.448
26	0.492
52	0.610
104	0.607

about 0.6 M. DTT up to 1 mM was also added. After 24 hours standing (including 10—15 hours at room temperature) the thick syrup-like mass was dialyzed for 72 hours against five changes of 2 liters of 1 mM tris-HCl, pH 8.5.

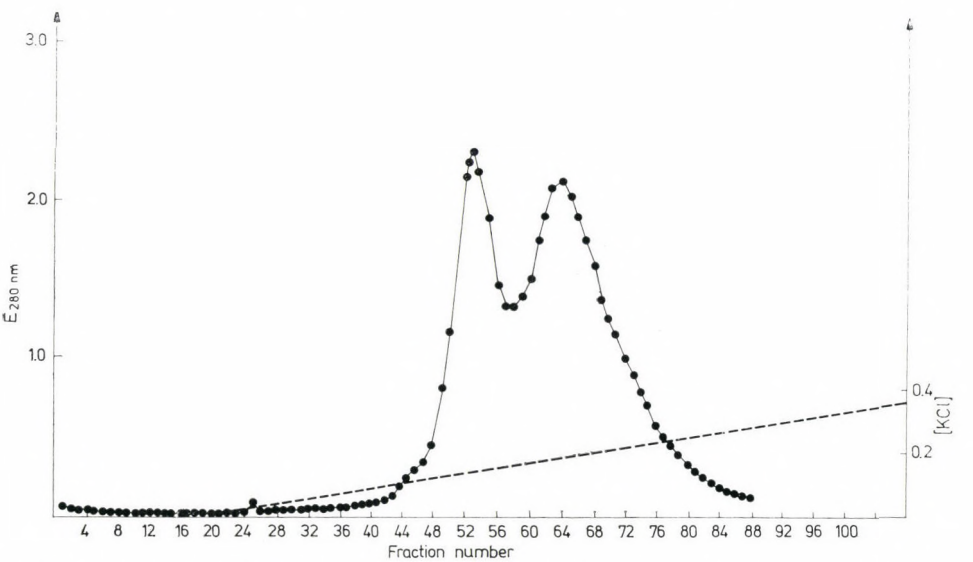

Fig. 1. DEAE cellulose chromatography of the ammonium sulfate fraction. On a 2× 25 cm Whatman DE-52 column, made up in 0.02 M Tris-acetate, pH 7.5, 200 mg protein (15—20 mg/ml) was chromatographed. After addition of the protein the column was washed with one bed volume of starting buffer, followed by a KCl gradient to 0.3 M. The flow rate was 18 ml/hour and 4.5 ml fractions were collected. Chromatography was carried out at 4 °C

The denatured protein mass was well mixed with an equal volume of the dialysis solution and centrifuged at 10 000 *g* for 30 min. The precipitate was washed once more as above, and the supernatants were combined.

The precipitate obtained from this crude extract at 30% saturation of ammonium sulfate (at 0 °C) was dissolved in 0.5 mM $KHCO_3$, 1 mM DTT, dialyzed overnight against the starting buffer used for DEAE chromatography and clarified by one hour centrifugation at 100 000 *g*.

The details of the DEAE cellulose chromatography giving the final homogeneous product are described in the legends to Fig. 1.

After chromatography we obtained 30–50 mg pure α-actinin.

In Fig. 1 we show the DEAE cellulose chromatography of the protein fraction from the crude extract precipitated by ammonium sulfate. As seen on the gel electrophoresis pictures in Fig. 2 e and f, both fractions consisted mainly of α-actinin, but the first peak eluting between 120–160 mM KCl was contaminated by components heavier than α-actinin and also by a relatively low molecular weight component. The second peak eluting between 170–200 mM KCl was pure α-actinin. (Note that the gel is heavily overloaded!). In ATPase and turbidity tests the activity of the material in the first peak approximated that of the pure protein.

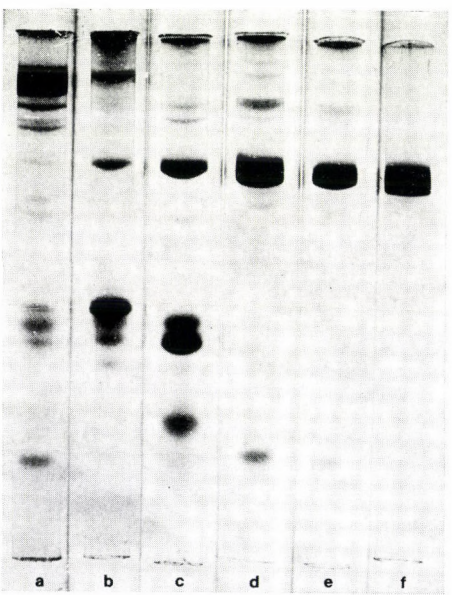

Fig. 2. Electrophoretic pictures of the different fractions obtained. a) Proteins extracted by Hasselbach-Schneider's solution. b) Proteins remaining undissolved after removal of $MgCl_2$ by dialysis and extraction by 1 mM Tris-HCl, pH 8.5. c) Combined supernatant obtained after removal of $MgCl_2$ by dialysis. d) Fraction of c) precipitated by 30% saturation with ammonium sulfate. e) First fraction (120–160 mM KCl) eluted from the DEAE cellulose column. f) Second fraction (160–200 mM KCl) eluted from the DEAE cellulose column (Pure α-actinin). 20 μg protein samples were electrophoresed in all cases

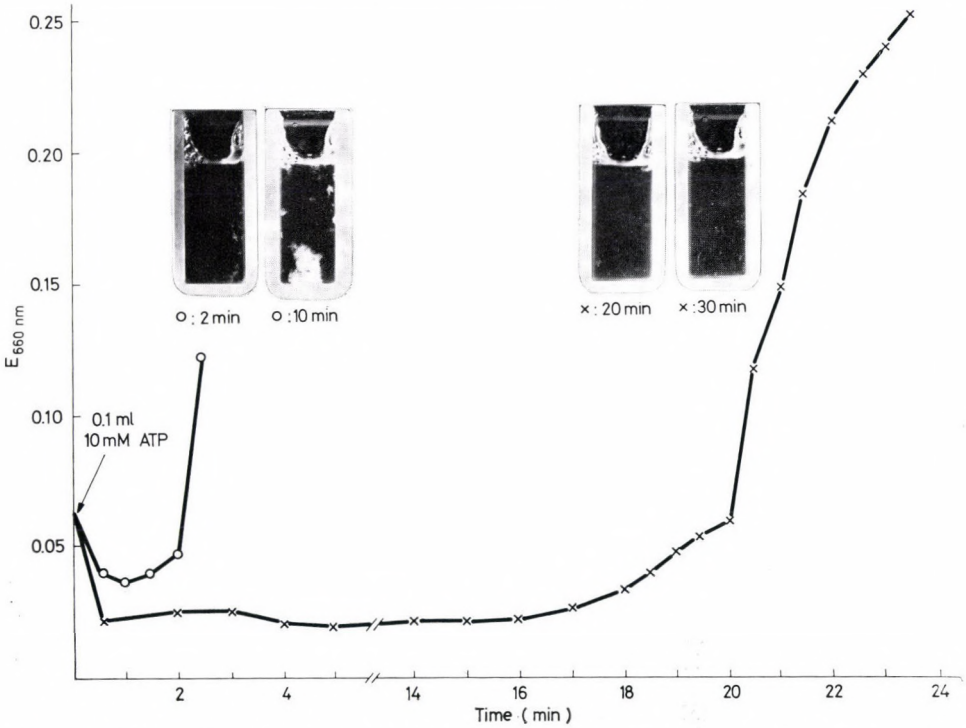

Fig. 3. Influence of α-actinin on the superprecipitation of actomyosin. 0.6 mg/ml actomyosin; 5 mM Tris-acetate, pH 7; 0.1 mM $MgCl_2$; 0.05 mM $CaCl_2$; 0.125 M KCl; 0.5 mM ATP. Test volume 2 ml. -------×-------×------- control; ------ ○--------○-------- 0.24 mg α-actinin added. Insets are photographs taken, at the times indicated, of the cuvettes containing the superprecipitation test mixtures with and without α-actinin.

On rechromatography the first slightly contaminated fraction did not give rise to more second-fraction material. The nature of the first fraction material remained obscure.

The pure α-actinin of the second peak was completely homogeneous in electrophoresis and sedimented as a single peak of 5.59 *S*. It exhibited all the activities characteristic of this protein (gelation of actin, enhancement of ATP-ase activity, acceleration of superprecipitation). In Table 1 the enhancement of actomyosin ATP-ase by our pure preparation is shown. In Fig. 3 we show the accelerating effect of our α-actinin preparation on the superprecipitation of actomyosin.

α-actinin prepared by the above procedure could be kept in 10% sucrose, 20 mM tris-acetate, pH 7.5, 1 mM DTT at −40 °C (Puszkin et al., 1977).

Our preparative procedure described here is more simple than the procedures described in the literature and it gives a satisfactory yield. According to preliminary experiments (Puzsár, 1975) tropomyosin in the extract obtained after the $MgCl_2$ denaturation procedure is fully active. Thus, this extract seems to be a favorable starting material for the preparation of this protein, too.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 223—228 (1980)

Studies on the Heat Inactivation of Porcine Kidney Aminoacylase

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(Received May 25, 1979)

Heat inactivation of porcine kidney aminoacylase (E.C. 3.5.1.14) was investigated under different conditions (i.e. at different temperatures, protein concentrations and pH values). Kinetic analysis of the inactivation process suggests that aminoacylase is a dissociable enzyme: the dimeric form is dissociated to enzymatically active monomers and the heat stability of the dimer is higher than that of the monomers. High temperatures, dilution and pH values other than neutral, all promote dissociation. The K_D^{app} at 60 °C, pH 7.0 is of the order of 10^{-6} M.

Introduction

Porcine kidney aminoacylase (E.C. 3.5.1.14) catalyzes the hydrolytic deacylation of acyl derivates of aliphatic L-amino acids, except derivates of L-aspartic acid, (Birnbaum et al., 1952) and also the cleavage of certain dipeptides (Rao et al., 1952, 1953; Fu et al., 1954). From a functional aspect, it is characterized by a high degree of stereospecificity (Birnbaum et al., 1952).

The enzyme molecule is a dimer composed of two monomers, each with a molecular weight of about 43 000 daltons (Kördel, Schneider, 1976).

We studied the heat inactivation of the enzyme and the analysis of the complex inactivation process reported here suggests that the dimeric enzyme is dissociated to catalytically active monomers.

Materials

Porcine kidney aminoacylase, a salt-free liophylized preparation was obtained from the Reanal Factory of Laboratory Chemicals, Budapest, Hungary. Its specific activity was 2500 ± 200 units/mg protein. In the calculations molecular weights of 86 000 and 43 000 were used for the dimeric and monomeric forms of the enzyme, respectively (Kördel, Schneider, 1976).

N-acetyl-L-methionine and other chemicals, all reagent grade, were obtained from Reanal, Budapest.

Methods

Determination of protein content: Protein contents were estimated by the method of Lowry et al., (1951) as modified by Schacterle and Pollack (1973).

Bovine serum albumin (Serva Fein Biochemica, Heidelberg, GFR) was used as standard for calibration.

Determination of aminoacylase activity: Aminoacylase activity was measured by the photometric method of Mitz and Schlueter (1958) using N-acetyl-L-methionine as substrate. The reaction mixture (3 ml) contained 0.03 mg/ml aminoacylase, 0.015 M N-acetyl-L-methionine and 0.1 M potassium phosphate, pH 7.0. One unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmole of N-acetyl-L-methionine in 1 hour at 25 °C, pH 7.0. Measurements were carried out in a Pye Unicam SP 1800 spectrophotometer equipped with a recorder.

Heat inactivation was carried out in 0.1 M potassium phosphate, pH 5–8, in 1 ml volumes. At different time intervals samples were withdrawn and quickly cooled in ice. The samples were filtered in order to remove denatured protein, and residual activity was determined in the filtrate (reaction time 2 min.). pH values were measured at the temperature of incubation.

Results

Temperature Dependence of the Inactivation of Aminoacylase

The temperature dependence of the heat inactivation of aminoacylase was studied in the range of 30–80 °C, at pH 7.0, the pH optimum of the catalytic activity. As shown in Fig. 1, the enzyme is fairly stable at 40 °C; at 60 or 80 °C, however, heat inactivation is no longer described by a single first-order reaction. A semilogarithmic plot reveals that two first-order reactions take place simultaneously, one faster and one slower. At 70 °C only the slower one proceeds at a measurable rate.

This phenomenon can be interpreted in two ways: (1) two forms of aminoacylase are present in the mixture, one of which is more stable than the other; (2) aminoacylase is dissociated and one oligomeric form is more stable than the other. In the first case the ratio of the two forms of aminoacylase must be a given value in the mixture, and therefore in the semilogarithmic plot the straight lines corresponding to the slower reaction should intersect the ordinate at an identical point independently of temperature, since the position of this point of intersection is determined by the ratio of the two enzyme forms in the mixture. However, Fig. 1 shows that at 60 °C the point of intersection is at 80%, while at 70 °C it is at about 55%, indicating that the system is not a mixture of two different acylases but a dissociable system.

The Dependence of the Heat Inactivation of Aminoacylase on Protein Concentration

The dependence of heat inactivation on protein concentration was studied at 60 °C, pH 7.0. Since dilution enhances dissociation and the dimeric form may be supposed to be more stable, one can expect that in dilute solutions the quantity of this form decreases. In this case the overall reaction will not be the sum of two,

purely first-order reactions, since active dimers and monomers are again and again equilibrated when inactive products are formed. In practice, considering the errors of the measurement, the semilogarithmic plot of the slower reaction is well approx-

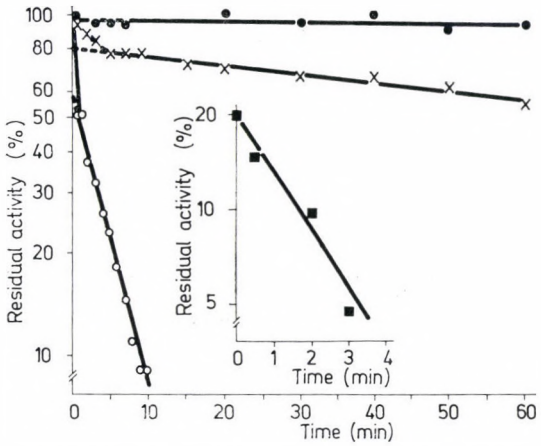


Fig. 1. Kinetics of the heat inactivation of aminoacylase at pH 7.0. The experiments were carried out in the presence of 0.1 M potassium phosphate. Protein concentration: 1 mg/ml. ●, 40 °C; ×, 60 °C; ○, 70 °C. The $t_{1/2}$ values of the slow reactions in the above order are: not measurable, 123 minutes and 3.5 minutes. The inset shows the kinetics of the fast reaction calculated from the experiment at 60 °C on the basis of the equation for parallel first-order reactions

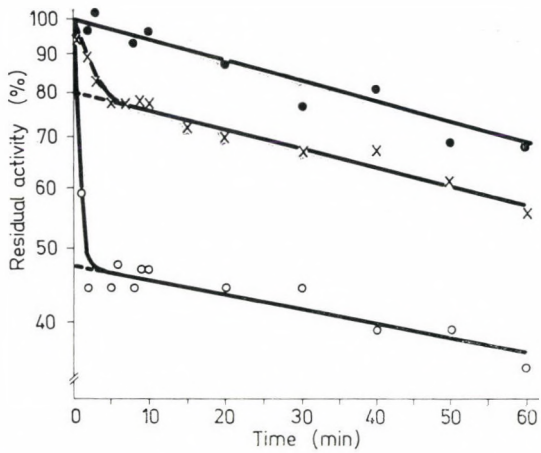


Fig. 2. The effect of protein concentration on the heat inactivation of aminoacylase at 60 °C and pH 7.0. The experiments were carried out in the presence of 0.1 M potassium phosphate. Protein concentrations and $t_{1/2}$ values; ●, 10 mg/ml, the $t_{1/2}$ of the slow reaction is 110 min; ×, 1 mg/ml, $t_{1/2}$ = 123 min; ○, 0.1 mg/ml, $t_{1/2}$ = 144 min

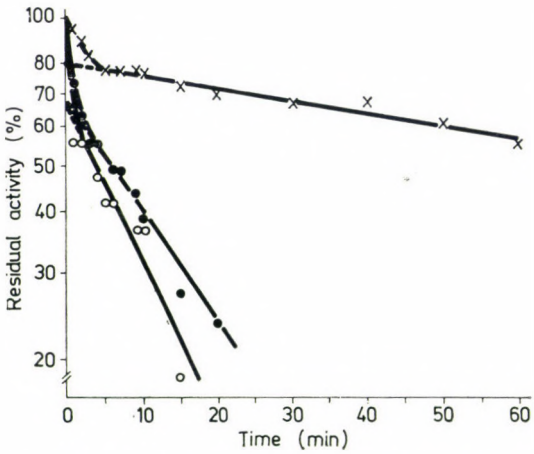


Fig. 3. The effect of pH on the heat inactivation of aminoacylase at 60 °C. The experiments were carried out in the presence of 0.1 M potassium phosphate. Protein concentration: 1 mg/ml. ●, pH 5.0; ×, pH 7.0; ○, pH 8.0. The $t_{1/2}$ values of the slow reaction in the same order are: 13.5 minutes, 123 minutes and 9.5 minutes

imated by a straight line. A typical series of experiments is shown in Fig. 2. From the data measured at starting enzyme concentrations of 1.0 and 0.1 mg/ml, we conclude that aminoacylase indeed is a dissociable enzyme and the dimeric form is considerably more stable than the monomer. For the more stable form, $t_{1/2} = 123 \pm 9$ min. This is in good agreement with the values measured in concentrated solutions (10 mg/ml). In the latter case, the reaction can be described by one first-order reaction; the concentration of the monomeric form is presumably so

Table 1

Distribution of the dimeric and monomeric forms of aminoacylase as a function of protein concentration at 60 °C, pH 7.0 (the mean of 5 heat inactivation experiments)

Starting enzyme concentration		Distribution (%)		Concentration ($M \times 10^6$)		K_D^{app} ($M \times 10^6$)
mg/ml	*$M \times 10^6$	Dimer	Monomer	*Dimer	**Monomer	
0.1	1.16	50.2 ± 1.2	49.8	0.58 ± 0.01	1.16 ± 0.02	2.32 ± 0.16
1	11.6	80.5 ± 1.1	19.5	9.34 ± 0.13	4.52 ± 0.25	2.19 ± 0.29
***10	116	(93.26)	(6.74)	(108.19)	(15.62)	(2.26)

* — calculated using the molecular weight of the dimer.
** — calculated using the molecular weight of the monomer.
*** — the values in brackets were calculated from the means of the K_D^{app} values determined in heat inactivation experiments using 0.1 mg/ml and 1 mg/ml solutions.

low that its inactivation could not be detected. From these results we also conclude that the dimer-monomer pre-equilibrium is fast and it does not affect the kinetics of heat inactivation.

As the intersections on the ordinate of the straight lines in Fig. 2 give an approximation of dimer and monomer concentrations in the enzyme solutions, we calculated the apparent equilibrium constant of the system by the following formula:

$$K_D^{app} = \frac{[\text{Monomer}]^2}{[\text{Dimer}]}$$

The data are listed in Table 1. It can be seen that the K_D^{app} values obtained for the 0.1 and 1.0 mg/ml solutions are in good agreement. On the basis of the mean values, in a 10 mg/ml enzyme solution the ratio of monomers amounts only to a few per cent and therefore monomers are untraceable in the heat inactivation experiments.

The Effect of pH on the Heat Inactivation of Aminoacylase

The pH dependence of the rate of heat inactivation was studied at 60 °C, at an enzyme concentration of 1 mg/ml. As shown in Fig. 3, the application of acidic or mildly alkaline media brings about an effect similar to that of raising the temperature, i.e. the dissociation of the dimer is promoted. Shifting the pH of the medium to either direction from pH 7.0 considerably decreases the stability of the dimer. For instance, the half life calculated for the heat inactivation of the dimer at pH 7.0, 123 minutes, decreases to 13.5 min at pH 5.0 and to 9.5 min at pH 8.0, which means that the reaction proceeds by about one order of magnitude faster.

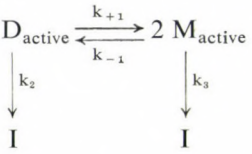
Discussion

Heat inactivation studies of native aminoacylase revealed that, instead of exhibiting the expected simple first-order kinetics, the inactivation process was of a more complex nature. Inactivation may be a complex reaction if (1) enzyme forms of identical function but of different stability (e.g. isoenzymes) are present in the system, if (2) in the course of the treatment protein isomerization occurs, or if (3) an oligomeric enzyme is dissociated and the oligomer and the monomers differ in stability (Fischer et al., 1973).

As porcine kidney aminoacylase has been shown to be a dimeric molecule (Kördel, Schneider, 1976), it seemed highly probable that the complex reaction observed by us is related with the dissociation of the dimer.

This presumption seems to be supported by the distinct dependence of the rate of heat inactivation on protein concentration. Accordingly, the partial processes of the complex reaction, i.e. the dissociation of the dimer into monomers

and the inactivation of dimers and monomers are described by the following scheme:



$k_3 > k_2$ and k_{+1} and $k_{-1} \gg k_2, k_3$

$\text{activity}_D = 2 \times \text{activity}_M$

where *D* indicates the dimer, *M* stands for the monomers and *I* for the inactive forms; k_2 and k_3 are the rate constants for the heat inactivation of the dimer and the monomers, respectively.

Owing to the interactions between the polypeptide chains, the dimer is more stable than the monomers, i.e. it is less susceptible to heat inactivation. The elevation of the temperature, the application of media with pH values different from neutral or the decrease of protein concentration, all shift the equilibrium in the direction of monomers, and in consequence the amount of the more stable dimer is decreased in the system. At high temperatures (70°C), in acidic or mildly alkaline media (pH 5.0 or 8.0, respectively) or at low protein concentrations (0.1 mg/ml) – under the conditions applied by us – it is only the denaturation of the dimer that proceeds at a measurable rate.

Our experiments indicate – and indeed it is implied in this interpretation – that the monomers of aminoacylase are enzymatically active, and that the activity of the dimer is equal to the sum of the activities of the monomers. In order to decide whether the monomeric form can be considered as the basic catalytic unit, further experimental work is needed.

The author is deeply indebted to Dr Gertrud Szabolcsi for her extensive help and valuable advice in the course of the preparation of the manuscript, and to Mrs Erzsébet Dala and Mrs Margit Zumpf for their excellent technical assistance.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 229—236 (1980)

Metal Content of Rat Liver Cell Organelles

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(Received May 25, 1979)

Zn^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+} were determined by means of atomic absorption techniques in tetramethylammonium hydroxide digests of cell organelles isolated from normal rat liver tissue. The metal content of the cell organelles was expressed with reference to their protein content. The results showed that digestion with tetramethy la mmonium hydroxide can be applied for the determination of metal to protein ratios in the cell organelles and that these ratios appear to be suitable for the characterization of cell organelles.

Introduction

An increasing number of observations suggest that metals play an important role in intracellular metabolic processes. Although it is beyond question that a better understanding of the biological effects of metals can be achieved primarily by the isolation and biochemical characterization of metalloprotein, i.e. specific metal-binding proteins, an important role must be assigned also to the quantitative distribution of metals in cell organelles.

Investigations on the metal content of cell organelles have been continued for quite a long time, yet, data in the literature are rather contradictory (Anghileri, 1976; Ghazi, Heaton, 1975; Hamilton, Holdsworth, 1975; Stonard, Webb, 1976; Yamaguchi, Yamamoto, 1975). While suitable methods of isolation, extraction and assay have become available by now, it is the basis of reference of the metal content of the samples that poses difficulties and results in conflicting reports.

The protein content of the sample as a reference basis seems to be the most appropriate parameter.

Murthy et al. (1973) proposed the use of TMAH for the solubilization of minced tissues. This method is advantegous as it permits a simultaneous assay in the solubilized tissue of the metal content by atomic absorption and protein according to Lowry et al. (1951). In a previous work (Ludány et al., 1978) we used TMAH to determine the total metal content of rat liver.

In the present paper we report on the measurement of Zn^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+} and Mg^{2+} levels by atomic absorption techniques from isolated cell organelles digested by TMAH. The intracellular metal content was expressed with reference to total protein content determined concomitantly.

Abbreviation: TMAH, tetramethyl ammonium hydroxide.

Material and methods

Livers of male albino (Wistar) rats, kept on normal diet and weighing 150–200 g were used. After decapitation and exsanguination the livers were perfused through the portal vein with 0.33 M sucrose (pH 6.0), at 4 °C. For a single experiment the livers of two rats were used. After weighing the excised and combined livers (8–10 g) they were homogenized in a tenfold excess (w/v) of the above non-buffered sucrose solution. Cell disruption was performed at 4 °C by a Tornado^R tissue blendor (Braun-Melsungen, GFR) at low speed. The efficiency of cell rupture was checked in a light microscope after staining the homogenate with a 0.1 per cent solution of crystal violet. After filtering the homogenate through a double layer of cheese cloth, the cell components were fractionated by centrifugation. Nuclei and nondisrupted cells were pelleted at 2800 rpm ($800 \times g$) for 15 min, mitochondria at 12 000 rpm ($10\,000 \times g$) for 20 min, and microsomes at 19 500 rpm ($40\,000 \times g$) for 60 min, in the S-34 rotor of a Sorvall (Du Pont USA) RC 5 centrifuge at 4 °C. Cytosol was obtained by centrifugation at 47 000 rpm ($100\,000 \times g$) for 60 min, in a VAC 601 (Janetzki, GDR) ultracentrifuge. In this way four sets of pellets were obtained following each centrifugation step. These pellets were separately analyzed as parallel samples. For the metal and protein determination the entire amounts of the pellets were used.

Depending on the quantity of the pellets as well as the expected protein content of the supernatants, 3–5 ml TMAH of the 10 per cent solution, as supplied by the manufacturer (Ferak, reinst GFR) were used for solubilization. Digestion of the samples was carried out in the centrifuge tubes for 12 hours at room temperature followed by 30 min at 65 °C. After cooling the homogenous samples were diluted with deionized water to a known volume. From these samples, henceforth, referred to as starting solutions, metal and protein determinations were made.

Measurement of metal ion content was performed in a Jarrel-Ash Type. 82–518 atomic absorption spectrophotometer (Fischer Co. USA) supplied with a laminar burner, in a mixture of acetylene and air.

For the measurements of Zn and Fe, the samples were further diluted with deionized water, whilst to analyze Ca and Mg dilutions were made with strontium chloride solution. To determine Cu^{2+}, the samples were not diluted, but directly injected in to the instrument. Solutions containing H_2O-TMAH and sucrose-TMAH in the proper dilution, served as blank solutions. Standard solutions (BDH Chemicals Ltd Poole England) treated in the same way as the samples served as reference.

From the metal and protein concentrations, the latter measured according to Lowry et al. (1951), μg metal/g protein values were calculated.

From the data on volume and concentration of the cell fractions we calculated the values for different metals and total protein of the cell and related these calculated values to the data obtained by direct measurement of the starting homogenate (recovery). Furthermore the distributions (percentage) of various metals and proteins of the cell among the different organelles were also calculated.

In the experiments extreme care was taken to avoid metal contamination. Except heat treatment we preferably used plastic vessels cleaned by Q–93 (Ferak, GFR) detergent and deionized water. Solubilized samples were not stored in glassware.

Results and discussion

In four series of experiments the metal and protein content of total tissue homogenates and the cell components from the combined livers of 2 rats were assayed.

1. Based on parallel measurements we determined the Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} content, as related to the protein in content of total liver tissue exsanguinated by perfusion (Table 1). Knowing the wet weight of the liver tissue, the metal and protein content was also expressed in terms of μg metal per gram tissue and gram protein per 100 g tissue (Table 2). The metal content of normal liver tissue, related to protein as well as wet weight, followed the order $Mg^{2+} > Fe^{2+} > > Zn^{2+} > Ca^{2+} > Cu^{2+}$. On the average, the standard deviation of the metal per protein ratios was found to equal $\pm 11\%$ of the mean (Table 1). As related to the wet weight of the perfused livers, the standard deviation from the mean was $\pm 17\%$. In both cases the highest standard deviation was observed for Mg^{2+}. It reached $\pm 23\%$ of the mean Mg per protein values measured in the four experiments.

2. To determine the metal to protein ratios of the cell organelles the following samples obtained from a single differential centrifugation step were used: the so-called crude nuclear fraction ($800 \times g$ pellet), mitochondrial fraction ($10\,000 \times g$ pellet), microsomes I ($40\,000 \times g$ pellet), microsomes II ($100\,000 \times g$ pellet) and cytosol ($100\,000 \times g$ supernatant).

a) The metal to protein ratios of cell organelles as derived from the data of four experiments are summarized in Table 1. While the values measured from parallel extractions in the same experiment differed from the mean by a mere 5% (data not shown in the Table), between different experiments, similarly to the total homogenate, a relatively higher standard deviation was found even in the case of the fractionated cell organelles. Again, the standard deviation was the greatest for Mg^{2+}. The Zn^{2+} and Fe^{2+} content of the fractions proved to be the most constant parameters.

Taking into account the mean values $\pm$ standard deviation of the various metals, the metal to protein ratios were highly variable within the cell. Thus, no Ca^{2+} was detected in the cytosol, while in the mitochondria and in the microsomes a twofold excess of Ca^{2+}, as related to protein, was found compared to the nuclear fraction. Only slight differences in the amount of Mg^{2+} could be observed among the various cell fractions. However, the Mg^{2+} to protein ratio was relatively the highest in the microsome fractions in each series of experiments. Fe showed a ten times higher metal to protein ratio in the $100\,000 \times g$ pellet than in other cell con-

Table 1

Metal content of cell fractions after solubilization with TMAH

Means ± standard deviations calculated on the basis of 4—4 determinations in four different experiments

	Ca^{2+}	Mg^{2+}	Zn^{2+} μg metal/g protein	Fe^{2+}	Cu^{2+}
Total homogenate	56.08 ± 5.08	1116.8 ± 256.8	170.6 ± 19.0	327.3 ± 33.8	22.16 ± 0.43
Liver cell fractions					
nuclei	60.62 ± 13.62	981.7 ± 357.6	120.2 ± 27.3	182.4 ± 68.2	18.62 ± 2.63
mitochondria	103.74 ± 20.31	1162.0 ± 395.7	113.1 ± 7.5	271.3 ± 23.4	18.27 ± 6.68
microsomes 40 000× *g*	101.00 ± 16.40	1461.9 ± 387.6	189.0 ± 23.2	280.1 ± 79.3	14.88 ± 1.68
microsomes 100 000× *g*	102.60 ± 12.10	1692.5 ± 501.7	300.8 ± 32.1	3194.3 ± 330.9	38.18 ± 0.53
cytosol	N.D.	976.0 ± 214.2	337.5 ± 38.3	271.0 ± 71.7	40.14 ± 1.00

Table 2

Percentage distribution of the metal and protein content in various fractions of the liver cell

Means ± standard deviations calculated from the data of four experiments

	Ca^{2+}	Mg^{2+}	Zn^{2+}	Fe^{2+}	Cu^{2+}	Protein
Total homogenate (μg metal/g wet tissue)	9.54 ± 1.36	190.08 ± 47.0	29.04 ± 2.48	55.71 ± 14.58	3.77 ± 0.54	* 17.02 ± 2.73
Liver cell fractions (distribution in %)						
nuclei	41.0 ± 7.7	36.2 ± 5.4	25.7 ± 2.7	21.3 ± 5.4	30.6 ± 4.4	40.2 ± 2.6
mitochondria	36.5 ± 7.2	22.3 ± 4.3	12.6 ± 1.6	16.5 ± 1.2	15.6 ± 6.3	20.9 ± 0.9
microsomes I.	16.1 ± 2.2	12.8 ± 2.6	9.6 ± 2.8	7.7 ± 1.0	5.8 ± 1.0	9.5 ± 1.3
microsomes II.	6.4 ± 1.5	5.7 ± 1.4	5.9 ± 2.0	34.3 ± 5.5	5.8 ± 1.4	3.7 ± 0.6
cytosol	N. D.	23.0 ± 6.3	46.2 ± 3.8	20.2 ± 4.4	42.2 ± 5.3	25.7 ± 1.9
Recovery from the fractions (μg metal/g wet tissue)	10.12 ± 1.48	185.48 ± 54.40	31.96 ± 3.78	58.63 ± 16.39	4.16 ± 0.56	*16.90 ± 2.80

* g protein/100 g wet tissue.

stituents. Our measurements also demonstrate that cytosol proteins are the richest in Zn^{2+} and Cu^{2+}.

b) On the basis of the wet weights of the liver tissues, the volumes of each cell fraction, and the metal and protein concentrations determined, we also calculated the intracellular percentage distribution of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} and protein, as well as the recoveries with reference to the values measured directly for the total tissue homogenate. This latter, however, could be done with only a limited accuracy owing to in accuracies involved in the volume determinations.

Data on recoveries and intracellular percentage distributions are listed in Table 2. On the basis of mean values the recovery of the individual components from the fractions ranged from 97 to 110 per cent. Based on the calculations, with the use of the isolation technique described under Materials and methods, 40.2 per cent of the total protein content of liver cells was found in the nuclear fraction, while this figure for the cytosol had a mean value of 25.7 per cent.

According to our results almost 80 per cent of the total Ca^{2+} content of the cells is localized in the cell nucleus and in the mitochondria (with mean values of 41 per cent and 36.5 per cent, respectively), while 36 per cent of the Mg occurs in the nuclear fraction. The cytosol contained 17 to 30 per cent of the total Mg^{2+}, with a relatively high standard deviation (mean 23 per cent). More than one-third (with a mean of 34.3 per cent) of the Fe^{2+} content of the perfused liver tissue was found in the cell fraction sedimenting at 100 000 $\times g$. The cytosol contained almost 50 per cent of the Zn^{2+} and Cu^{2+} (46.2 and 42.2 per cent, respectively).

Digestion of organ and tissue samples by TMAH enabled us to determine simultaneously the metal content by atomic absorption as well as the protein content by Lowry's procedure (Murthy et al., 1973; Ludány et al., 1978).

Digestion with TMAH proved to be a highly suitable method for measuring the metal content of cell organelles, for two reasons. First, because the recovery of metal ions and protein from the individual fractions ranged between 97 and 110 per cent and second, because in parallel measurements the metal to protein ratios showed a less than 5 per cent standard deviation from the mean value. *The Zn^{2+}, Cu^{2+}, and Mg^{2+} values obtained by us for total liver tissue* showed a good correlation with those reported in the literature (Bremner et al., 1973; Burch et al., 1975; Kang et al., 1977; Ohtake et al., 1978; Riordan, 1975; Stonard, Webb, 1976). The amount of Ca measured in a sucrose-containing medium after ashing was found to be higher than that found by us (Anghileri, 1976; Burch et al., 1975; Ghazi, Heaton, 1975). A possible explanation of this discrepancy may be the ubiquitous contamination of sucrose by Ca^{2+}. Data concerning Fe^{2+} content have been reported so far only for non-exsanguinated (Kang et al., 1977; Powanda et al., 1978).

Homogenization of cells in 0.33 M sucrose at pH 6.0 seems to be especially suited for metal ion determinations since at the low pH applied the cell nuclei preserve their structural integrity even in the absence of Ca^{2+} and Mg^{2+}. This is also indicated by the high protein (40 per cent) as well as the high Ca^{2+} and Mg^{2+} content of the nuclear fraction, and by the fact that in the cytosol no Ca^{2+} could

be detected and the relative Mg content did not exceed 30 per cent. Our previous analyses had shown that the Mg^{2+} content of the cytosol rose above 30 per cent of the total Mg^{2+} content of the cell only if additional Ca^{2+} was added to the medium used for cytosol preparation (Ludány et al., 1978).

Characterization of individual cell components on the basis of their different metal to protein ratios may serve as a useful tool in cell biology. The intracellular distribution of a given metal is not uniform: in certain cell fractions it is enriched while in others its proportion to protein is very small. Thus, the different values may refer to the intracellular localization of metalloproteins. On the basis of our data individual cell fractions are characterized by definite metal ion to protein ratios. In the *crude nuclear fraction* all the assayed metals can be detected, with the exception of Mg^{2+}, their proportion to protein differs from that characteristic of the cytosol. It is considerably lower for Zn^{2+}, Fe^{2+} and Cu^{2+} while it is much higher for Ca^{2+}. These differences may reflect not only the heterogeneity of the complexes (e.g. the relatively higher share of other protein complexes) but also the strength and stability of metal-proteins bonds in the cell nucleus. The metal-protein bond is presumably looser in the case of Mg^{2+}, since the Mg^{2+} to protein ratio was nearly the same in all cell constituents examined.

Mitochondria contain almost 40 per cent of the total Ca^{2+} of the cell. The Ca^{2+} to protein ratio does not exceed significantly that of the microsomes.

The *microsomal* ($100\,000 \times g$) fraction is characterized by an exceptionally high Fe^{2+} to protein ratio. In addition, comparatively high levels of Zn^{2+}, Cu^{2+} and Mg^{2+} occur in this particular cell fraction.

Relatively numerous data are available concerning the metal and protein content of the *cytosol.* However, due to differences in the isolation procedure, these data are sometimes contradictory. Thus, depending on the method used to disrupt the cells 25 to 60 per cent of the total protein content of the cell was found in the organelle-free fraction (Anghileri, 1976; Smeyers-Verbeke et al., 1977). According to some authors Ca^{2+} to protein ratio of the cytosol is fairly high (Anghileri, 1976). In contrast to this, we could not detect Ca^{2+} by atomic absorption in either our present, or our previous analyses of the cytosol prepared by the sucrose isolation technique described.

Yamaguchi was able to detect Ca^{2+} binding protein in the $100\,000 \times g$ supernatant obtained from sucrose homogenates of liver cells only by means of isotope tracer techniques (Yamaguchi, Yamamoto, 1975). In our previous analyses using an ultrafiltration procedure, some Mg was found in a non protein-bound state, while in the case of Zn^{2+}, Fe^{2+} and Cu^{2+} the existence of metal-protein bonds was confirmed (Ludány et al., 1978). Our measurements of the distribution of Zn^{2+} and Cu^{2+} inside the liver cell agreed with those of others who similarly found nearly half of the Zn^{2+} and Cu^{2+} content of the cell in a protein-bound state in the $100\,000 \times g$ supernatant (Bremner, Davies, 1976; Bremner et al., 1973; Chen et al., 1975; Ohtake et al., 1978; Riordan, 1975; Smeyers-Verbeke et al., 1977; Wang, Pierson, 1975). On the basis of the results presented our method seems suitable for the investigation of cell constituents and, by determining metal to protein

ratios, for the characterization of different cell fractions. Under appropriately controlled condition of isolation and fractionation it may also be used for the detection of fine intracellular changes in the metal to protein ratios in model experiments of pathological significance.

This work was supported by the Scientific Research Council, Ministry of Health, Hungary.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (3), pp. 237—244 (1980)

Zn-Binding Protein Profile of Rat Liver Cytosol.

Cromatographic and Electrophoretic Study

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$^{65}Zn^{2+}$ was given to rats per os and intraperitoneally for in vivo labelling of Zn^{2+}-metalloproteins of cytosol. At the chromatographic analysis of the cytosol, two $^{65}Zn^{2+}$-containing protein peaks were found in per os, while an additional low molecular weight third peak appeared in the intraperitoneal dosing samples. The chromatographic fractions were further separated by a non-denaturing gradient polyacrylamide electrophoresis technique.

At the electrophoretic analysis of the chromatographic fractions the recovery of $^{65}Zn^{2+}$ was the highest in samples taken from a second chromatographic peak (88%), which indicates the presence of proteins with exceptionally strong Zn^{2+}-binding character in the 30—40 000 molecular weight fraction of the cytosol.

Introduction

In living cell the organelle-free cytosol is quite abundant in Zn^{2+}. On the basis of our earlier ultrafiltration analyses the Zn^{2+} seemed to be nonfiltrable indicating that zinc associates tightly to proteins of cytosol in vitro and probably inside the living cell too (Ludány et al., 1978).

In spite of the rapidly growing interest in specific Zn^{2+}-binding proteins (Gurba et al., 1972; Harvey, Barry, 1975; Kissling, Kägi, 1977; Riordan, 1976) only preliminary efforts have been devoted to the systematic analysis of intracellular Zn^{2+}-binding proteins (Norheim, Steinnes, 1975; 1976). The lack of analysis of Zn^{2+}-binding protein spectrum in living cells might mainly be related to methodological difficulties.

It is hard to make an appropriate analysis of intracellular proteins without their denaturation which consequently alters the relationship between metals and protein molecules.

In the present study the Zn^{2+}-binding proteins were labelled in vivo with $^{65}Zn^{2+}$. The advantage of our approach is that the Zn^{2+}-protein complexes are first fractionated by column chromatography and the chromatographic fractions further analyzed with a new non-denaturing gradient electrophoresis technique. The $^{65}Zn^{2+}$ isotope was given to rats in two different ways: per os and intraperitoneally. The Zn intake of the animals was higher in case of intraperitoneal than in per os dosing and also higher than the normal Zn^{2+} supply of the control animals.

The increased Zn^{2+} intake induced a third low molecular weight peak in the chromatographic pattern of Zn^{2+}-binding proteins.

At the electrophoretic analysis, a few protein bands with exceptionally high $^{65}Zn^{2+}$-binding activity were found.

Materials and methods

Two groups of four male albino rats weighing 150 to 170 g were exposed to $^{65}Zn^{2+}$ isotope.

Rats of the first group were daily injected intraperitoneally with 10 μCi $^{65}Zn^{2+}$ isotope (Hung. Acad. of Science Isotope Department, Budapest; Specific activity: 0.14 Ci/g). The total amount of daily injected Zn was about 70 μg/animal. The isotope administration was continued for 14 days. The rats of the second group were also given 10 μCi $^{65}Zn^{2+}$ isotope daily for 14 days in their food instead of intraperitoneal injections. Both groups of animals were kept on normal diet.

The animals were killed by decapitation 24 hours after the last isotope exposition. The livers were perfused through the portal vein with 0.33 M sucrose solution. The pH of the unbuffered sucrose solution was always less than pH 6. Livers of four animals were combined in both experimental groups, and homogenized in fresh 0.33 M sucrose at 1 : 10 Vol/Vol ratio with a Tornado* homogenizer (Braun-Melsungen, GFR). The homogenate was centrifuged first at $800 \times g$ for 20 minutes then the supernatant was further centrifuged at $100\,000 \times g$ for 1 hour. The $100\,000 \times g$ supernatant was used as cytosol in this study. The ultrafiltration experiments were carried out in a standard pressure cell (AMICON, Lexington USA) using UM-2 filter and nitrogen for pressure. The samples were dialyzed against three times changed 0.1 M Tris-Glycine buffer (pH 8.3) at 4 °C for 24 hours.

The gel chromatograpy was carried out at 4 °C on column (15 × 900 mm) of Sephadex G-75 Fine (Pharmacia Ltd, Uppsala Sweden) with 0.01 M Tris-HCl buffer (pH 7.2) supplemented with 0.1 M NaCl. The flow rate was 1 ml/min and 3 ml fractions were collected. The protein concentration in the starting material and also in the eluated fractions was either estimated by UV absorption at 280 nm or by the Lowry procedure (Lowry et al., 1951). The radioactivity of $^{65}Zn^{2+}$ in the fractions was estimated in a gamma counter (LKB) Wallac Ultro-Gamma 1280).

For electrophoresis, we used an increasing exponential gradient of 3-to 20% polyacrylamide gel in a Tris-Glycine (pH 8.3) buffer system, which has been proved to be a non-denaturing condition for proteins (Barrowman et al., 1973; Cherian, 1974; Davis, 1964). Twenty, 9 cm long glass tubes with 6 mm inner diameter were filled with the gradient gel at once. The chromatographic fractions selected for electrophoresis were combined and concentrated in an Amicon pressure cell to a protein concentration of 3–5 mg/ml. For studying the protein pattern, the same amount of proteins (150 micrograms/tube) and for the analysis of $^{65}Zn^{2+}$ distribution the amount of the proteins applied to the gels changed according to the spe-

cific activity of the samples (See Fig. 3.). The run was continued for 3 hours at 3 mA/tube with a Constant Current, Constant Voltage Power Supply (Buchler Instr. New Jersey USA).

After finishing the run, the glass tubes were carefully broken up and the gels either stained with Amido Black or sliced for counting. Starting from the top of the gels 3 mm slices were cut and placed separately in plastic vials and counted for five minutes. In each cases the recovery of the applied counts was also estimated in the upper gel segments where the protein bands were located.

Results

The amount of $^{65}Zn^{2+}$ isotope in the cytosol samples of the two experimental groups was different (Table 1.).

On the basis of the ultrafiltration analysis with UM-2 filters the $^{65}Zn^{2+}$ seemed to be nonfiltrable in both experimental groups. In accordance with the ultrafiltration analysis, the specific radioactivity of the cytosol samples did not change during a 24-hour dialysis in 0.1 M Tris-Glycine buffer (pH 8.3) at 4 °C.

At the chromatographic analysis of the cytosol two $^{65}Zn^{2+}$-containing peaks were found in the per os and three in the intraperitoneal samples (Fig. 1.). The first peaks were eluted in the void volume and consequently they represented Zn^{2+}-binding proteins with molecular weight (MW) higher than 70 000. The second peaks corresponded to a MW of approximately 30 – 40 000. The third peak was eluted from the column in the 32 – 35th fractions that is in 95 – 105 ml elution volume, which corresponded to a molecular weight of about 10 – 12 000 daltons.

Table 1

*^{65}Zn Radioactivity in Cytosol of Per os and Intraperitoneally Treated Rats**

Sample		Protein mg/ml	$^{65}Zn^{2+}$ cpm/mg protein
$^{65}Zn^{2+}$ per os	100 000 g supernatant	4.6	360
	UM-2 concentrate	14.6	350
	UM-2 ultrafiltrate	—	non detectable
$^{65}Zn^{2+}$ i.p.	100 000 g supernatant	4.65	1850
	UM-2 concentrate	12.00	1830
	UM-2 ultrafiltrate	—	non detectable

* Details are in "Materials and methods".

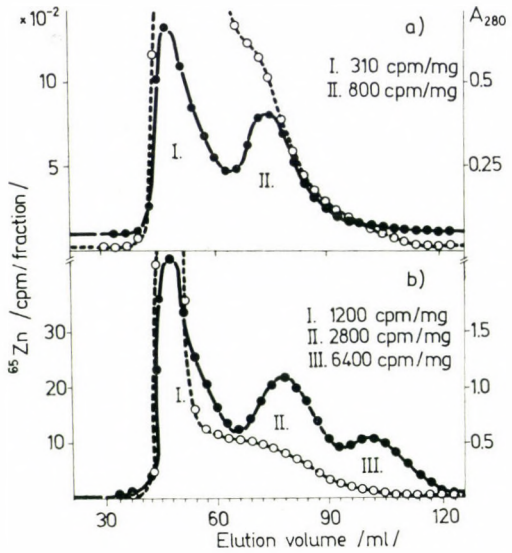

Fig. 1. Sephadex G-75 column chromatography of the liver cytosol isolated from rats pretreated with ^{65}Zn per os (a) and intraperitoneally (b). The preparation of the cytosol is described in Materials and methods. Sephadex G-75 was packed in a Pharmacia K 15 column (90× 1.5 cm) and equilibrated with an elution buffer (0.01 M Tris-HCl, 0.1 M NaCl, pH 7.2). The flow rate was 1 ml/min. and 3.0 ml fractions were collected at 4 °C. The amount of proteins chromatographed was 29 mg in a., and 24 mg in b. samples. ————, ^{65}Zn radioactivity; — — — —, absorbance at 280 nm

This third peak found only in the intraperitoneal samples had the highest $^{65}Zn^{2+}$/protein ratio. After 120 ml elution there was no more $^{65}Zn^{2+}$ found in the fractions. The recovery of the radioactivity applied to the columns was 90–95%.

The electrophoretic pattern of the same chromatographic fractions was similar, therefore only the pattern of three peaks of an intraperitoneal sample is shown here in Fig. 2. The recovery of the isotope in gel segments corresponding to the protein bands was higher (88%) in the second than in the first (57%) and third (45%) chromatographic peaks. Furthermore three well defined protein bands with exceptionally high $^{65}Zn^{2+}$ content were always found in samples of the second chromatographic peak (Fig. 3.).

Discussion

The repeated administration of $^{65}Zn^{2+}$ isotope at a low dose resulted a remarkable labelling of Zn^{2+}-binding proteins of rat liver cytosol. The specific labelling i.e. count per mg protein was higher in case of intraperitoneal than in per os dosing samples. In both cases the Zn^{2+} was in a nonfiltrable, protein-bound state. In addition to the difference in specific labelling, a difference was found in

the chromatographic pattern too. Only two peaks were present in per os dosing while an additional third peak appeared in the intraperitoneal dosing samples (Fig. 1). The approximate molecular weight of this third $^{65}Zn^{2+}$ peak is about 10–12 000 daltons which corresponds to the size of metallothioneins. The absence of the low molecular weight Zn^{2+}-binding metallothioneins in per os and their appearance in the intraperitoneal dosing samples correlate well with findings of Richards, Cousins, 1975; Bremner et al., 1973; 1976; Davies et al., 1973; and Chen et al., 1977.

At the intraperitoneal dosing experiments the total Zn^{2+} intake should exceed the limit of Zn intake which regularly induces an elevation of hepatic Zn^{2+}-metallothionein content (Burch et al., 1975; Davies et al., 1973; Kang et al., 1977; Richards, Cousins, 1976).

Nevertheless, the relationship between Zn^{2+} supply and hepatic metallothionein content has not been clarified yet. It is still a question whether the en-

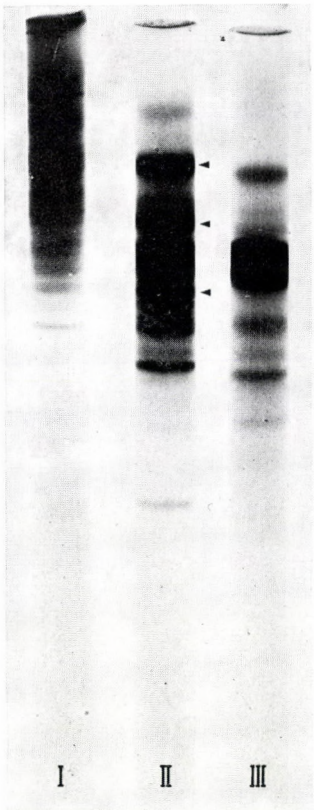

Fig. 2. Exponential gradient polyacrylamide gel electrophoresis of protein samples taken from chromatographic peaks of Fig. 1. b. (I. II. and III). The amount of the proteins applied to the gels was 150 μg in each sample. The gels were stained with Amido Black

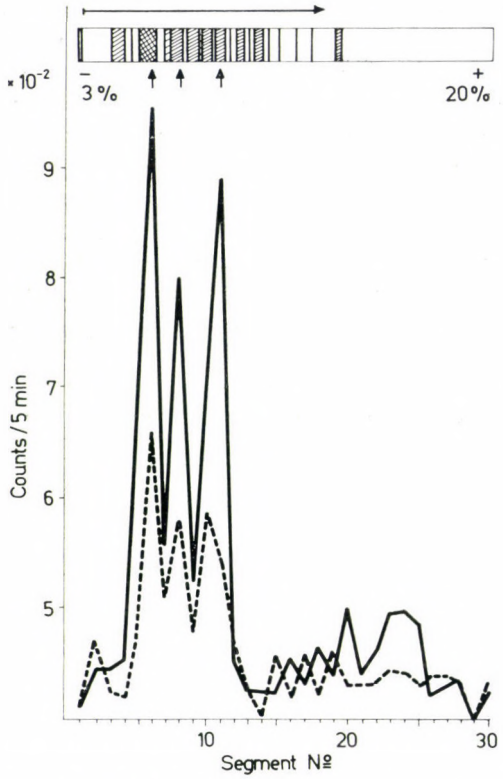

Fig. 3. Distribution of the ^{65}Zn radioactivity after acrylamide gel electrophoresis of sample II. presented in Figure 2 (————); and the same second chromatographic peak taken from liver cytosol of rats treated with ^{65}Zn per os (— — — —). The counts and the amount of protein applied to the gels are the followings: (————) 2800 counts/5 min. in 200 μg protein; (— — — —) 1600 counts/5 min. in 400 μg protein

hanced Zn intake causes a promotion of the de novo synthesis or only a stabilization of hepatic Zn^{2+}-metallothioneins (Feldman, Cousins, 1976).

The exponential gradient of polyacrylamide gel and the Tris-Glycine buffer was found to be a good running condition for electrophoretic analysis of Zn^{2+}-binding proteins. We could get a few sharp protein bands from each chromatographic fractions and a significant amount of $^{65}Zn^{2+}$ remained attached to protein molecules. The selection of the running conditions in these experiments was based on a series of previous electrodialysis experiments where a wide spectrum of different buffers were analyzed (unpublished). It was found that acidic medium with and without urea used in a certain type of one- and two-dimensional polyacrylamide gel electrophoresis techniques and 0.1 % SDS with and without urea used in other type of one- and two-dimensional electrophoresis always disturbed the protein-metal interactions (Kellermayer, Busch, 1973; O'Farrell, 1975; Orrick et al., 1973;

Panyim, Chalkey, 1969; Shapiro et al. 1967; Weber, Osborn, 1969). The bulk of Zn was released from cytosol protein molecules at a few mA current intensity in the above-mentioned media. On the other hand if the electrodialysis was carried out in a Tris-Glycine buffer at a slightly alkaline pH, more than half of cytosol Zn^{2+} (70%) remained in protein-bound state.

At our running conditions the protein bands always located in the upper two thirds of the gel. As we were interested in the distribution of $^{65}Zn^{2+}$ in the electrophoretically separated proteins, only the upper 6 cms of the 9 cm long gel rods were analyzed for radioactivity. Consequently, the recovery of the applied counts could really indicate the association character of Zn^{2+} to different proteins of the chromatographic fractions. Surprisingly, a remarkable difference was found in Zn^{2+}-binding capability of proteins of the three samples taken from the three peaks of the chromatographic separation. The lowest $^{65}Zn^{2+}$ recovery was found in case of the third peak although this sample had the highest specific activity and was the most homogeneous for proteins. As we have discussed above, this third chromatographic fraction may contain metallothioneins, or metallothionenin-like Zn^{2+}-binding proteins.

The Zn^{2+}-protein interaction was the strongest in the second, middle molecular weight peak. Three distinct protein bands with exceptionally high ^{65}Zn content were found in this sample, while such kind of proteins has not been revealed in samples taken from the highly heterogeneous first and the low molecular weight third peaks.

At this moment we can not identify these strong Zn^{2+}-binding 30–40 000 molecular weight proteins. Their further characterization is in progress now in our laboratory.

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Book Reviews

Gel chromatography Theory, Methodology, Application. by Tibor Kremmer and László Boross.
Akadémiai Kiadó, Budapest, 1979.

The ever increasing claim for more and more powerful separation methods has met with the arising importance of the isolation of biopolymers and this is reflected in a growing number of applications of gel chromatography.

Only in 1979, the references on Pharmacia-produced gel filtration materials comprise a total of 1000 citations. At the same time, the basic books about gel chromatography were published about 10—12 years ago. From this point of view the book by Kremmer and Boross supplies a long felt need.

The book consists of three main parts (besides the preface, historical survey and acknowledgements) on 299 pages. The chapters and sections are numbered according to the decimal system.

Part I (THEORY) surveys the practical fundamentals of gel chromatography and the basic theoretical considerations. The practical fundamentals of gel chromatography deal with the structure of gels, the gel forming materials and the commercially available gels — among them the Hungarian made Molselect and Acrylex gels. The second segment of Part I is theory in its narrow sense that applies to gel chromatography: the geometric, volumetric, steric, etc. models; selectivity, efficiency and capacity factors; definition, significance and use of several gel chromatographic parameters. Some of the well-known practical and theoretical rules introduced are explained here. The influence of flow rate on HETP, the effect of temperature, adsorption, concentration of sample solutes, etc. on resolution. These 55 pages of the book (pp 48—103) are not only a good critical evaluation of the theory of gel chromatography but the references are up to date, the figures are clear and the theoretical considerations and rules are really well understandable.

Just a minor problem is caused by the different uses of abbrevations. The publisher of the Hungarian version of the book insists on some special symbols (for instance peak width is w in both English and German texts but b in books printed in Hungarian; the void volume is V_0 in any book or brochure except the Hungarian ones in which it has to be V_k). These symbols remained unaltered on pp 70—75 and some other parts, although in general the book follows the correct use. It is especially disturbing that the author's own investigations become less understandable in consequence of such odd abbreviations. On the other hand, the unusual interpretation of HETP does not cause confusion, only alteration from the well known chromatographic books.

Part II (METHODS AND TECHNIQUES) is really necessary for anybody working with gel chromatography. Informations on the selection and preparation of gels, on particle size, preservation and sterilization of gels, selection of chromatographic columns, on column size, wall effect, tubing and joints, etc. are useful and valuable for everyone whether a beginner or specialist. Inclusion of some other topics (regulation of flow rate, fraction collectors, etc.) completes this part. In Chapter 3 of Part II some special techniques are dealt with in detail: recycling gel chromatography, solubility chromatography, zone precipitation, desalting and buffer exchange, thin-layer gel chromatography, etc. Ion exchanger gels as well as

their applications are not discussed in the book.

Part III (APPLICATION OF GEL CHROMATOGRAPHY) consists of six chapters:

1. Application in protein chemistry,
2. Gel chromatography in the chemistry of nucleic acids,
3. Gel chromatography of carbohydrates,
4. Gel chromatography of synthetic polymers,
5. Gel chromatography of small molecule organic compounds,
6. Gel chromatography of inorganic ions.

The authors took the best examples from the practice and the figures illustrate a wide range of applications. Some particularly interesting separations as well as the optimalizations of gel chromatographic parameters (e.g. temperature, flow rate, pH, etc.) are presented. This chapter describes the most significant methods and examples developed earlier and recently alike and deals with the minimum of theory.

Some of the abbrevations are also taken from the Hungarian version of the book without adequate retranslation (R instead of R_s = resolution, b instead of w on page 246, etc.). In this part the authors fail compare gel chromatography with alternative preparative methods (ion exchange chromatograhy, ion exchange gel chromatography, affinity techniques) and the place of gel chromatography among other preparative methods has not been outlined either. Similarly, other possible versions of analytical procedures (ultracentrifugation, molecular size determination by PAGE or SDS-PAGE, etc.) are also missing from the book. These non-essential deficiencies are probably the result of the limited volume and narrow scope of the book.

The book is based on the Hungarian edition of Gel chromatography (Gélkromatográfia, Műszaki Könyvkiadó, Budapest, 1974). This original edition has been revised and expanded with the most recent results of gel chromatography. The book is a much needed addition to the literature of gel chromatography that helps us learn this powerful technique. The theoretical rules are well explained, the practical methods to be used are presented in great detail. The very few "retranslation mistakes" do not interfere with the great informative value of this book to organic chemists, biochemists and analytical chemists at any level.

H. KALÁSZ

The Chemistry of Radiopharmaceuticals by N. Heinde, H. D. Burns, T. Honda and L. W. Brady (editors), Abacus Press, Abacus House, Tunbridge Wells, 1978 294 pages.

This book is one of the Cancer Management '78 series. The aim of the series is to provide practising physicians with as much information as possible on the possibilities of rapid diagnosis and prognosis of malignant tumorous lesions.

The text of the introduction is based on the material of a symposium held on April 23–24, 1976 in the Hahnemann Medical College, Philadelphia, USA. The literature includes articles published up to the end of 1977.

The authors state, and this is indeed so, that the science of radiopharmacology has undergone a tremendous development during the past years, and accordingly the number of medical examinations and treatments by the use of labelled compounds has risen markedly. Since the individual chapters are linked only by what has been mentioned in the introduction, we consider it best to discuss these chapters one by one.

The first chapter deals with the past and the future of nuclear medicine. A brief and useful survey is given. Mass-production of radioactive substances is designated as a turning-point in the history of nuclear medicine (preparation of labelled substances in a nuclear reactor was achieved by Fermi in 1942!).

Chapter 2 demonstrates that virtually all studies of receptor binding sites and correlations between drug structure and function have involved labelled substances in recent years. The functions of the liver, spleen and the reticuloendothelial system (RES) can readily be examined by using insoluble colloids (^{198}Au, ^{111}In and $Fe_2O_3-Fe(OH)_3$). Further, particles with dimensions larger than those of colloids permit the scintigraphy of the small vessels. Examination of iodine uptake by the thyroid by means of ^{131}I was elaborated before the Second World War. The other procedure that can now be said to be a classical one is the examination of amino acid uptake by the pancreas.

Currently, cationic ^{99m}Tc (pertechnetate 99m) is used most widely for studies of the RES. ^{99m}Tc is bound by the organs comprising the RES (90% in the liver) 5–10 minutes after injection. ^{99m}Tc bound in various complexes may also be employed for studying capillary function. Examinations are carried out with ^{51}Cr, ^{197}Hg, ^{203}Hg or ^{81}Rb to detect cell damage. Many isotopes have been tested and are suitable for the localization of cardiac infarcts. In addition to the negative pictures (the labelled substance cannot enter the damaged tissue), positive fluorescence can be attained with ^{99m}Tc tetracycline chelate, for instance, at the site of the infarct. Later, the author deals with localizations based on biochemical observations. For example, by the use of ^{131}I steroids adrenal tumors can be localized, with polycyclic labelled dyes (e.g. Rose Bengal) liver and bile duct filling can be examined, etc. Besides successful localizations based on biochemical mechanisms, some unsuccessful cases are also described. Finally, the chapter discusses some positive and negative results based on pharmacological observations, e.g. ^{67}Ga citrate and bleomycin chelates can be utilized for tumor localization, etc.

Chapter 3 deals with the requirements labelled drugs have to fulfil. The purity requirements depend on the area of use. For instance, labelled substances employed for therapeutic purposes have to fulfil different requirements than those used in in vitro studies.

The author tabulates the half-lives and the photon and beta-emission energies of the most important substances used in diagnostics and therapeutics. Attention is drawn to the fact that, in the case of good positron cameras and the construction of cheap medical cyclotrons, the use of positron-emitting isotopes will continuously gain ground in diagnostics.

It must be mentioned that even at the beginning of the book there is very much repetition, in both the text and the literature references. This should somehow have been eliminated by the editors, as it only unnecessarily increases the bulk and the price of the book. As an example, at least those references could have been omitted from the general review which deal with the diagnostics of particular organs, whose diagnostics and therapy are being treated in a separate chapter. One such chapter is that on the pancreas. For instance, references 12 and 33 in Chapter 3 are indentical, and some important references are not listed.

Chapter 4 deals with the radiodiagnostics and therapy of the pancreas. The author feels that this is a difficult and still "empty" field. The difficulties of ^{75}Se-selenomethionine examinations are treated first. In the case of the pancreas, in spite of the many examinations to date, nuclear medical methods are not satisfactory for differential diagnosis. A number of efforts appear promising, e.g. examinations with labelled amino acids, thioglucose complexes, etc.

Chapter 5 discusses the requirements radiopharmaceuticals that are commercially available and in the experimental stage have to fulfil. It states that, under the present conditions, the quality control of labelled drugs used for therapeutic purposes is of such a high standard that it excludes the possibility of contamination or secondary transformation. A section on "matter-antimatter" interactions deals with the possibilities of the pharmaceutical application of positron-annihilation radiopharmacology and the positron camera. These questions have already been touched upon in an earlier chapter on future perspectives. The author and the editors see the future of medical diagnostics in the application of the cheap cyclotron and the similarly cheap positron emitter tomographs. This is the reason why the following section also deals with the positron-emitting nuclei and with the possibilities of their incorporation into various radiopharmaceuticals. In the future, preparations such as amino acids labelled with ^{11}C and ^{13}N and ^{18}F-labelled deoxyglucose are expected to become of wide use. As regards such compounds, Chapters 14 and 16 return to the application of ^{18}F-5-fluoro-2'-deoxyuridine, ^{11}C-labelled carboxylic acids and their derivatives as radiopharmaceuticals and diagnostic agents. The latter substances can be employed for in vivo pharmacokinetic studies of pharmaceuticals. The proton-emitting labelled pharmaceuticals are important non-destructive diagnostic agents.

The activities of the Brookhaven National Laboratory in particular are connected with the above work.

Subsequently, the authors deal with the preclinical evaluation of radiopharmaceuticals. A detailed description is given of the selection of the objects for animal experiments, and of the different metabolic characteristics of various laboratory strains. Examples are presented of the techniques involved in the administration of labelled compounds, the significance of the choice of solvents, the manner of sacrificing animals, *in vitro* and *in vivo* stability and toxicity studies, etc.

Many articles in the 1975 volume of J. Nuclear Med. (and also its subsequent volumes) report on the use of ^{99m}Tc. Three of the earlier chapters, Chapters 9, 13 and 17, deal with this topic. Besides these chapters, even the section describing the activities of the Brookhaven National Laboratory turns to the medical nuclear chemical applications of pertechnetate, an important element in Group VII of the Periodic System. Another element in this group is rhenium, which is similarly used for diagnostic purposes, in the form of ^{188}Re. Of the elements in Group III, the uses of ^{67}Ga and ^{111}In are dealt with, but the more recently employed ^{57}Co is omitted. It is a fact, however, that the various derivatives of ^{99m}Tc can still be used most advantageously for diagnostic purposes.

Two more chapters must be referred to. One of these is on the use of ^{125}I-labelled fibrin in the diagnosis of deep venous thromboses. This section presents a very good example of how a long-used isotope can be applied in increasingly new diagnostic areas.

A very short chapter deals with the evaluation of color prints emphasizing the advantages of evaluating color rather than black-and-white pictures in the application of radiopharmaceuticals in nuclear medicine. The possibilities and the value of standardizing the evaluation of color prints are also discussed.

Looking at the book as a whole, one can say that the aim of the editors, to give a survey of the most recent problems of nuclear medicine in a given country, the U.S.A., has been achieved, but only up to 1978. Consequently, because of the flood of recent results during one year, many of the chapters have virtually completely lost their interest and significance.

B. MATKOVICS

Prostate Gland und Seminal Vesicles. Handbuch der mikroskopischen Anatomie des Menschen. Vol. VII./6 by Gerhard Aumüller, 380 pages, with 142 partly colored figures, 181 separate illustrations.
Springer Verlag, Berlin—Heidelberg—New York, 1979

The male accessory sexual glands, especially the prostate gland of the rat, have been regarded for a long time as useful models by biochemists and endrocrinologists interested in the molecular mechanism of androgen action. Biochemists working in this field have often made attempts to discuss their data from the point of view of relationship between structure and function and to match biochemical data with morphological changes. Therefore, it may be interesting for them to see in the present volume how the morphologist combines into one picture the biochemical and morphological results available at present. The author has also included a number of information from the fields of molecular endocrinology, physiology and even pathology which make the descriptions more versatile. Looking at the complexity and the heterogeneous cellular composition of these organs one problem becomes increasingly evident, namely, in most cases the techniques in general use do not differentiate among these functionally different cell types.

In the first chapter the histogenesis and organogenesis of the prostate gland and the seminal vesicle are dealt with. Among others a concise yet comprehensible section is written about the spontaneous intersexual states that represent special models for biochemical-genetic investigations. The second and third chapters are devoted to the micro- and macroscopic as well as the functional morphology of the prostate gland and the seminal vesicle, respectively. Both chapters consist of sections written on the epithelium, the connective tissue and musculature, the blood vessels and innervation.

The human, canine and rat prostates are discussed in detail. The reader can find a coordinated and excellent presentation of morphological and biochemical results. Examples for this are the treatment of the functional morphology of the prostate and the informative description of the secretory

products and the mechanism of secretion in both accessory sexual glands.

In contrast to the relative abundance of data on the mechanism of hormonal regulation, the scarcity of biochemical information about the mechanism of neuroregulation of these organs is striking. Similarly, it is shown that while insight into the hormonal regulation of the epithelium is fairly deep, a lot of research is still needed to reach a similar level of knowledge on the hormonal regulation of the other tissues present in the organs in question. What may be stimulating for the fantasy of the biochemist is the description of well characterized morphological phenomena the biochemical basis of which is poorly understood. An example for this is the appearence of specific autophagic vacuoles in the accessory sexual glands after castration of adult rats or mice.

The volume presents a great number of micrographs of very good quality. Investigations carried out using a variety of histological techniques including freeze fracture methodology, latex cast and fluorescent techniques are reported.

The coupling of the up-to-date morphological data with the most relevant biochemical findings will expectedly contribute to a successful filling of the gap between the efforts of morphologists and biochemists. This book is concise, well constructed and contains many references. It deserves a place on the book-shelf of the investigator looking into the intricacies of the structure and function of the accessory sexual glands as well as of those specialists whose work, in some way or another, is related to these interesting organs.

M. TÓTH

Monographs on Endocrinology: Glucocorticoid Hormone Action by J. D. Baxter and G. G. Rousseau (eds), Springer-Verlag, Berlin—Heidelberg—New York, 1979.

In the last decade glucocorticoid hormones have become of common interest to researchers working in very different fields. Anybody who wants to deal with the action of glucocorticoids sooner or later will have to turn to biochemistry, endocrinology, cell biology, genetics, pharmacology or therapeutics. This monograph puts together results from the above-mentioned fields. The editors give a comprehensive review on the most important results of the glucocorticoid hormone-studies born in the last 15 years. The editors made a thorough selection of the relevant papers, collecting 34 of them by the best experts in this field.

The monograph first deals with the effects of glucocorticoids on different target tissues and cells in vivo as well as in tissue cultures. In further chapters details of the mechanism of hormone action are given. Characterization of the glucocorticoid receptor and approaches to its purification are summarized next. Two further chapters deal with factors influencing the stability and the nuclear- and DNA-binding capacity of the receptor. The most interesting problems of glucocorticoid hormone action, such as the precise mechanism of binding of the hormone-receptor complex to chromatin and DNA and induction of mRNA-synthesis, have not yet been settled. Recent results and new theories concerning these problems are discussed. Several chapters deal with the action of glucocorticoids on normal and leukemic lymphocytes, with the immunosuppressive and antiinflammatory effects as well as the influences of glucocorticoids on brain function, differentiation and hepatic metabolic processes. Last but not least the authors emphasize the fact that the understanding of glucocorticoid action means more than that of hormone action in general: it is also an approach to the regulation of gene expression in higher organisms.

In the reviewer's opinion the greatest advantage of this monograph is that if brings together a large body of information scattered in various journals and gives a coherent view of our present knowledge of glucocorticoid hormone action. It certainly will prove very useful to endocrinologists, biochemists, cell biologists, pathologists, pharmacologists as well as medical students working in this field.

A. NÁRAY and I. HORVÁTH

Membrane Transport in Biology by G. Giebisch, D. C. Tosteson and H. H. Ussing. (eds) Vol. I.: Concepts and models, 1978, 537 pp.; Vol. II.: Transport across single biological membranes, 1979, 443 pp.; Vol.

III.: Transport across multimembrane systems, 1979, 459 pp.; Vol. IV. A. and IV. B.: Transport organs, 1979, 939 pp. Springer Verlag, Berlin—Heidelberg—New York.

In recent years a considerable number of extensive monograph series have been published which appeared at irregular intervals or different places yearly, and whose publication was then protracted over a period of several years. These four volumes comprising some 1900 pages practically appeared simultaneously, which means that the whole of the material presented in this series is based on the critical evaluation of the latest data on the respective subject. The majority of the citations refer to works published up to 1976.

The three editors have divided the subject of membrane composition and transport into 55 chapters. These were compiled by over 60 authors following a common concept.

All those who are just beginning their studies on membrane transport, or who are interested in some special aspects of transport processes may find highly instructive, clearly written chapters covering nearly the entire field of our present knowledge of biomembranes. The individual chapters offer, in the first place, an extensive survey of the physical and physiological aspects of membrane transport, and lay due emphasis upon the biochemical mechanisms involved.

In volume I fundamental aspects of membrane transport are summarized, such as theoretical basis of investigations concerning active transport processes across biological membranes, some methodological aspects most frequently encountered in membrane biology and chemical and biological properties common for the majority of biological membranes. Finally the scope and limitations of lipid bilayer membranes widely used as biological membrane models are reviewed.

The chapter concentrating on passive transport, after giving some fundamental definitions, provides information about the statistical and mathematical treatment of the relevant processes. The chapter on the interpretation of tracer fluxes presents the concept and use of isotope tracers as non-perturbating tests. After a mathematical review of the nonequilibrium thermodynamics of isotope flow across membranes, its application to model systems is described. Similarly, methodological aspects dominate in the chapter dealing with the measurement of membrane potential with microelectrodes. After surveying the lipid and protein composition of erythrocytes, a genetic background of membrane composition in microorganisms, higher organisms and cultured somatic cells is presented. A brilliant chapter is devoted to correlations between ion transport and ATP formation, including the Mitchell hypothesis on chemiosmotic coupling the role of ATPase in the functioning of the proton pump, correlations between the function of sarcoplasmic Ca^{++} transport and Ca^{++}-activated ATPase, and between monovalent cation transport and $Na^{+} + K^{+}$-ATPase are also discussed. The chapter on the immunological reactions of membranes describes investigations concerning antibodies against ATPases, immunological reactions of the outer membrane surface and of lymphocytes and tumor cells. Experience of ever growing importance is presented in the chapter concerned with membrane receptors, cyclic nucleotides. Particular emphasis is laid on transport interactions.

The three final chapters provide a detailed survey — over 150 pages — of the properties of artificial lipid membranes, carrier-mediated transport across lipid membranes, finally of channels in black lipid films. The second volume of the series comprises 10 chapters dealing with transport processes across single biological membranes separating the inside and outside of cells or organs. It is not by mere chance that 4 chapters are devoted to the transport processes of red cells, the most extensively studied object.

The first chapter presents, along with very detailed data concerning the water permeability of red cells, an extensive review of the transport of nonelectrolytes, i.e. sugars, amino acids, purines and nucleotides. The three following chapters provide excellent information about the ion transport of erythrocytes. This is probably one of the most comprehensive summaries published in membrane monographs on the subject during the past few years. Gunn, author of the chapter on anion transport in erythrocytes, points out — among others — that the rapid movement of physiologically very important anions, chloride and bicarbonate, across red cell membranes is mediated by a system which

permits the anion exchange through the membrane about 10,000 times faster than the rate of charge transport by anions through red cell membranes.

In the chapter introducing the passive cation fluxes of red cell membranes Lew and Beaugé lay special emphasis on the role of specific interaction between membrane components and ions in the passive ion transport processes requiring no energy. The authors draw attention to the so-called "Gárdos-effect". that is to the Ca^+-sensitive K^+-permeability mechanism in the red cell membrane. In their chapter dealing with the active ion transport processes of red cells, Sarkadi and Tosteson, beside characterizing the Na and K pump of blood cells, describe the enzymatic mechanism of the process and some of the cellular functions of the Na/K pump. The same chapter also contains detailed and well constructed information about the Ca^{++} transport of erythrocytes.

After summarizing the ion transport processes of the axon, the most extensively studied nerve-cell membrane, the sodium, potassium and chloride movements across the plasma membrane of the frog skeletal muscle are dealt with. Scarpa's report on the processes occurring across mitochondrial membranes is focussed on H^+-transport. Following a description of the permeability of mono- and divalent cations, he gives a detailed survey of the transport of anions, metabolites and adenine nucleotides. The structural organization of the transport across the sarcoplasmic reticulum in skeletal and cardiac muscle is outlined by Inesi. This chapter summarizes the data concerning the binding, transport and release of Ca^{++}. The two final chapters of the volume provide a comprehensive survey of transport processes across the lysosomal membranes and chloroplast envelopes, respectively.

The contributions of the third volume are concerned with transport phenomena in multimembrane systems and in single epithelia. The volume includes chapters of a general nature on e.g. multimembrane systems in general, role of tight junctions in epithelial function, morphological aspects of transport, as well as chapters on specialized membrane systems such as those in giant algal cells, amphibian skin, amphibian urinary bladder, insect excretory epithelia, and insect gut epithelium. Two chapters cover transport in eye epithelia including the cornea, cristalline leans and ciliary epithelium. Separate chapters concentrate on ion transport across the choroid plexus, the sweat glands and the lacrimal gland. Volumes IV A and B summarize the properties of directional solute and solvent movements across complex epithelia; that is, the transport phenomena are discussed at the organ level. In most chapters the kidney tubules and the gastrointestinal tract are treated. Advances in the techniques have greatly contributed to the significant progress in this area. The review on the transport of water and solutes across the capillary endothelium is followed by chapters dealing with the electrochemistry of the nephrons and the electrophysiology of the kidney, respectively. Renal sodium, potassium and H^+ movement, as well as theoretical and practical aspects of Ca^{2+}, Mg^{2+} and P_i transport are discussed in detail. During the last years substantial progress has been made in studies concerning the mechanism of renal reabsorption of sugars, amino acids and weak acid buffers, as well as the secretion of organic acids and bases. This is excellently reflected in the appropriate chapters. After describing the principles and techniques of micropunction of the isolated nephron segments, their transport processes are discussed. The relation between active tubular transport and metabolism is the topic of another brilliant chapter. Here the influence of the metabolic activity of the cells on the passive transport properties of the epithelia is also underlined. Very accurate and extensive information can also be found on transport processes of water, electrolytes and proteins in the three major salivary glands of mammalian vertebrates, as well as on the mechanism of salt secretion by salt glands of nonavian vertebrates. In the chapter on gastric secretion particular emphasis is laid, using biophysical, electrophysiological and biochemical approaches, on the molecular mechanism by which H^+, Na^+, Cl^- and water are transported across the gastric epithelium. The reviews on the small and large intestine are based on our current understanding of the characteristics and physiological consequences of low-resistance paracellular pathways, water and electrolyte transport, as well as the colonic transport of weak electrolytes — ammonia and volatile

fatty acids. A splendid review deals with the transport of small solutes in the exocrine pancreas and the processes triggering electrolyte and enzyme secretion. The gall-bladder was the first tissue in which transepithelial transport was shown to occur in the absence of a sizable spontaneous transepithelial electrical potential difference. In the chapter on gall-bladder recent experimental results are summarized that have increased our knowledge about the mechanism of salt and water transport by this tissue. This chapter deals also with experimental procedures and theoretical considerations. The last chapter concerns the liver cells. The membrane of hepatocytes exhibits unique biophysical properties, especially with regard to passive permeability, facilitated diffusion and the electrogenic activity of the sodium pump. The characteristics of these different transport processes are reviewed with special reference to Na^+, K^+, Ca^{2+} and Cl^-, since these have been studied most intensively.

In short, the series of "Membrane Transport in Biology" is a useful glimpse of what is happening in this field and will be of considerable interest to investigatory concerned with all aspects of cell membranes and membrane transport. It provides a useful survey of research as well as guidelines for many aspects of future research in membrane biology. As a multipurpose source of up-to-date information and references this series should be available in libraries.

J. SOMOGYI

The Epstein-Barr Virus, by M. A. Epstein. B. G. Achong (eds), 72 figures, 29 tables, 460 pages. Springer Verlag, 1979, Berlin—Heidelberg—New York.

The Epstein-Barr virus (EBV), the first human cancer virus, was discovered 16 years ago and an immense body of information has so far been accumulated on it. This book gives the first over-view of all aspects of the virus in 19 chapters written by 20 contributors.

The EBV was found in an unusual human lymphoma first reported in 1958 by an unknown surgeon, D. Burkitt, in Uganda. After a long series of experiments the Burkitt lymphoma (BL) was successfully established in culture by Epstein and Barr who also found virus particles within the cells. This discovery, the general biology and the morphology of the virus as well as the virus-induced cytophathologic changes are described in Chapters 1 and 2. Serological studies showed that the EBV is related to the agents of three similar diseases: infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. EBV-associated antigens as membrane antigen, early antigen, viral capsid antigen, etc. were detected. It has become established that the virus belongs morphologically to the herpes group. These studies pointed to a world-wide dissemination of the virus (Chapters 3 and 4). Biochemical characteristics of the virus and its various effects at the cellular level, including cell-transforming activity of the viral DNA are summarized in Chapter 5. The EBV-genome is integrated into the DNA of the cell. Nucleic acid hybridization techniques were used in studies of viral DNA integration and genome homology (Chapter 6). The viral genome has also been detected in cells by biochemical means and heterogeneity of EBV isolates in various cell lines seems to be possible (Chapter 7). EBV-transformed cells contain different numbers of EBV genome copies, varying from a few to around a hundred per cell, but the number is characteristic of each individual cell line (Chapter 8). In chapters 9 and 10 presently available information on the events leading to transformation, and on the in vitro transformation process is reviewed. Classification and characteristics of various lymphoid cell lines are amply described in Chapter 11. The viral genome in lymphoblastoid cell lines can be activated either spontaneously or by chemicals, *e.g.* by IUdR and BUdR (Chapter 12). The role of EBV in the etiology of infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma is considered in Chapters 13, 14 and 15. The ability of the virus to induce malignant transformations under *in vivo* conditions, *e.g.* in various primate species and the significance of experimental carcinogenicity by the EBV are also discussed (Chapter 16). The EBV is compared with other oncogenic herpes viruses of both cold-blooded and warm-blooded animals (Chapter 17). Demographic studies carried out to show the role of EBV in the causation of Burkitt's lymphoma and prospective studies

for an epidemiologic approach to nasopharyngeal carcinoma are described in Chapter 18. Efforts made to produce vaccine capable of preventing EBV infection are surveyed in Chapter 19. This book is useful and of great interest to experts, teachers and students engaged in cancer research, virology, immunology, molecular biology, epidemiology, and cell culture.

I. PÁLYI

Enzyme Histochemistry. A Laboratory Manual, by Z. Lojda, R. Gossrau and T. H. Schiebler. Springer-Verlag, Berlin—Heidelberg—New York, 1979. 339 pages.

As a result of the rapid development of enzyme histochemistry during the last three decades an overhelming abundance of methods and their variations have piled up. Practice and critical revision have made a strong selection among them and a part of even the time-proven techniques can only be used for special problems or in special circumstances. Only a few experts can master this body of technical knowledge and give reliable recommendations for the general practice. This has been done by the authors of this book in a very appropriate way, as far as light-microscopy is concerned.

The general preparation techniques as well as the histochemical staining methods included in this volume are sublimated from the experience of two independent, renowned histochemical laboratories, in Prague and in Würzburg. Neither the specimen preparation techniques nor the staining prescriptions follow their individual original description but represent a consistent, uniform concept.

The book begins with a short chapter of general considerations including terminology, classification and reaction principles in a very concise form.

The next chapter describes a system of alternative tissue preparation techniques with sufficient details to be reproduced. This system covers, in addition to more conventional procedures and variations of fixatives, solvents and sequence of events, the handling of freeze-dried cryostat sections, incubation with gel media and the semipermeable membrane technique, and gives them their appropriate rank among the well-established techniques proposed for general use. The individual processing schemes are marked with abbreviations listed again on the back cover with the page number of their detailed description. For every detection method the alternatives for pretreatment are marked with these abbreviations. This system makes the correspondence between pretreatment and enzyme reactions unequivocal and makes the use of the book very practical.

The greatest part of the book deals with the individual detection methods, personally tried and recommended by the authors. For each enzyme considered there is a short summary of the reaction catalyzed, the biochemical properties and the physiological importance of the enzyme. The description of all methods follows a uniform pattern. For a number of enzymes more than one method is recommended with the discussion of their potentialities, and the preferences among them. The richest section is that about the hydrolases, a subject of preference in the activity of all the authors. Here, the methodological achievements of the very last years are included as well-established, reliable methods (e.g. peptidases). Another merit of the chapter is the system of prescriptions for dehydrogenases with the use of two stock solution (one for liquid and one for gel media) common for every enzyme.

The book is supplemented with some general practical information, e.g. preparation of buffers and a list of suppliers.

The structure of the book, and the description of the individual methods are clear, perspicuous, definite and simple so as to suggest as if enzyme histochemistry were one of the easiest disciplines to study. An illusion as this may be to some extent, it is sure that this manual can serve as a solid basis for the organization and reliable functioning of a contemporary laboratory for enzyme histochemistry. With its economical considerations and puritanism as regards the laboratory equipment this book proves at the same time that a considerable part of the contemporary and well-proved histochemical methods can be carried out with little special technical apparatus and under relatively modest material conditions. Hence, it may help making histochemistry a common property generally available for applied sciences, e.g. for routine medical pathology.

E. BÁCSY

Lehrbuch der Histologie, by H. Knoche. Springer-Verlag, Berlin—Heidelberg—New York, 1979. 427 pages.

This textbook of histology is primarily meant for the training of medical students. The author has laid emphasis on didactics by the clear structure of the text in which three levels of information are sharply distinguished by typographical means: one of a detailed textbook, one of the minimal criteria to be matched by medical students according to the actual subject syllabus in the GFR, and a condensed summary of each paragraph intended to help memorization before examinations. A didactically very important element of the book is the set of illustrations consisting of tables of schematic drawings uniform throughout the whole volume. The drawings, using a moderate degree of simplification, are well understandable and may be at the same time a good mediation for the elementary study of microscopic preparations.

The book covers the fields of cytology, histology, *s.str.* and microscopical anatomy of the organ systems.

The first chapter describes the structure of cells and cell organelles and provides some basic knowledge of cell kinetics and metabolism. A detailed subchapter is devoted to cell division.

After a short exposition of the notion of tissue and of cell contacts in general, the individual types of tissue (epithelial, glandular, interstitial, supporting, muscle and nerve tissues) are treated in full detail.

The remaining 60% of the text is the description of organ systems (blood, circulation, lymphatic, respiratory and digestive organs, urinary and genital systems, endocrine glands, central nervous system, sensory organs, and the skin).

A very short chapter (7 pages) containing some very basic information on light and electron microscopy, the principles of histological preparation, staining and histochemistry, is added.

The *Lehrbuch der Histologie* attempts to combine classical knowledge of microscopical histology with the more recent achievements of electron microscopy as well as with the most important functional features that characterize the individual morphological constituents. The main strength of the book is the classical component. The reviewer feels that the information from these three sources is not always successfully brought together under a homogeneous scope but sometimes represents a mosaic. This often leads to superfluous repetitions, in some cases to a lack of relation between fragments or even contradictions. An example for a less successful synthesis is the paragraph on adenohypophysis. Another critical notice is that the different chapters are not always at the same level of up-to-dateness. The achievements of the last fifteen years in cytology are rather incompletely assimilated. For instance, definition of GERL completely lacks the criterion of cytochemical differentiation. Though the most common histochemical features are mentioned now and then throughout the different chapters of the book, adequate histochemical information is missing at very important places (e.g. it is not even mentioned that endocrine cells secreting the individual peptide hormones in the hypophysis and elsewhere can be unequivocally identified by immunocytochemistry both in the light and in the electron microscope, hence some of the cell classifications have become obsolete).

Despite these critical remarks, the book presents the mass of basic information necessary for understanding the microscopic and submicroscopic structure of the human organism in a well-systematized, comprehensive way. The style of the text, the complete system of well-designed schematic figures as well as the esthetic appearance of the book contribute to making histology a pleasant subject to study.

E. BÁCSY

Renal Calculus by L. N. Pyrah, Springer-Verlag, Berlin—Heidelberg—New York, 1979., 370 pages, 55 illustrations.

In this book a profound and detailed picture is given on the theory of formation and on the biochemical and clinical aspects of renal calculi. In the introductory chapter the author describes the geographical distribution of nephrolithiasis and examines the role of nitritional as well as etiological factors. In the Second Chapter the theories of stone formation are discussed: according to one

of them the formation of the collodial matrix is the initial event followed by the deposition of inorganic crystals. According to an other one, the primary factor is the precipitation of crystalloid materials that occur in the urine as a result of hypersaturation depending on the pH and other factors. To support these assumptions the author provides a detailed account of the chemical, optical and crystallographical properties of calculi with different chemical compositions. His statements are illustrated by excellent microphotos. Histological plates of excellent quality demonstrate the changes of the stone-producing kidney, the position of the primary stone granules in the renal matrix and the alterations in the microstructure of the concremented kidney.

As far as crystallurias are concerned, the metabolid changes that result in the accumulation of stone-composing materials in the urine, are discussed in several chapters.

A detailed analysis of primary and idiopathic hyperoxaluria, disorders in the synthesis of oxalate, alterations in purine, phosphoris and magnesium metabolism and other biochemical processes makes this book particularly interesting for those involved in the thereotical aspects of nephrolithiasis. Simultaneously, the author describes those metabolic deseases in which the formation of calculi is only a part of the pathological process that affects the entire body. The treatment of the subject is very fortunate as the biochemical and the practical clinical informations are parallelly discussed. The chapter surveying the developmental disorders of the kidney and the urinary tract, that facilitate stone formation as a consequence of hidrance in urine transport, is very useful for the practitioner. From the chapter dealing with therapy, the possibilities as well as the limitations of dietic and drug treatments can be learnt. The book contains a chapter on operative surgery. Indication of renal and urethral stone surgery and the description of the surgical methods are fully adequate, containing well-founded conclusions based on experiences obtained on a large number of patients. The X-ray photos and the surgical illustrations are again excellent.

All in all: this book was written by an expert equally well experienced in both fundamental biochemical research and clinical practice related to nephrolithiasis.

The book is clear and well arranged in structure and proportions. The printers' work is also excellent.

M. VANIK

Bradykinin, Kallidin and Kallikrein in Handbook of Experimental Pharmacology, Vol. XXV, Supplement by E. G. Erdős (ed.), Springer Verlag, Berlin – Heidelberg – New York, 1979, 817 pages.

This volume of the series is to update and to complete the 1970 edition. The book consists of seventeen chapters and detailed subject and author indexes.

Chapter I gives an excellent historical review of the present knowledge of the interrelationships of the kinin system with other components of the blood. The kinin system of blood plasma is very complex and some questions of its interrelationships are still unanswered. The central role of factor XII has been determined. Through its activation three cascading and interrelated systems are set into motion: the intrinsic clotting, the fibrinolytic and the kinin-forming systems. Kallikrein, the main kinin-forming enzyme is liberated from prekallikrein by factor XII and exerts a positive feedback on factor XII. To date, no physiological role has been attributed to kinins with certainty. However, they are believed to play a role in pathological mechanisms.

Chapter II describes the alternate kinin-generating systems and the kininogenases of blood cells. The leucokinin-generating system has been studied in detail. The pharmacologically active leucokinins consist of 21 to 25 amino acids. Their liberating enzymes which can be inhibited by pepstain, are of lysosomal origin.

In addition to plasma kallikreins many organs produce another group of kallikreins. Chapter III tries to create an order in the field of these glandular kallikreins of mammalian origin. The pancreatic, salivary and urinary kallikreins are of similar nature. Small peptide substrates have been used for studying these anzymes.

Chapter IV reviews the kallikrein inhibitors of different origin. In this field the most valuable development comes from studies of the bovine pancreatic trypsin inhibitor

(BPTI) which consists of 58 amino acid residues and contains three disulfide bridges. Availability of BPTI of high purity stimulated its thorough conformation analysis and gave a possibility for its examination as a macromolecular enzyme substrate in complexes with trypsin and chymotrypsin. Synthesis of BPTI and its analog gave peptides with full inhibitory potency and several models of the reactive site of the molecule were synthesized. Furthermore, the experiments with kallikrein inhibitors have contributed to a deeper understanding of the mechanism of enzymatic processes.

Chapter V is an outstanding review on structure-activity relationships of analogs of bradykinins and bradykinin potentiating peptides. The knowledge of these relationships may contribute to a more conscious planning of agonist, antagonist and more active potentiating peptides and to a clearer understanding of their activation by enzymes and of their interaction with receptors. However, one should keep in mind that comparison of analogs tested in different biological assays is often extremely uncertain.

Chapter VI gives an account of the hypotensive kinins found in nature, i.e. bradykinin and its derivatives occurring in man and mammals, the venom kinins of wasps, yellow jackets, hornets and amphibian skin, tachykinins and putative kinins. Most of them are of known chemical structure.

The chapter on bradykinin receptors describes direct binding studies, modifications of receptors and responses in vivo and in vitro, receptor-effector coupling, structure-activity relationships and some theoretical approaches.

In chapter VIII the problems of radioimmunoassay of bradykinin are summarized: preparation of the subjects, preparation and preservation of samples, introduction of antibodies, labeled antigens and reaction conditions.

Chapter IX describes the effects of bradykinin on the nervous system. In the peripheral nervous system the role of kinins in inducing pain has lied to studies of interactions with prostaglandins, and nonsteroidal anti-inflammatory agents and to determinations of alterations in neuronal pathways as a result of i.a. administration of kinins. There is a growing interest in the elucidation of direct actions exerted by kinins within the central nervous system.

In chapter X the effects of kinins on the cardiovascular system, lung, gastrointestinal function, and reproduction are reviewed and inflammation and their possible role in exercise and stress are discussed.

Chapter XI gives a review of great use on the release of vasoactive substances by kinins. Bradykinin-prostaglandin interactions are discussed in detail, and release of cathecolamines and histamin is concisely summarized. Some experiments seem to prove that antidiuresis exerted by bradykinin is mediated by vasopressing.

Chapter XII dealing with kinases is one of the most valuable parts of this book. In the last ten years an immense development took place in this field. Kininase I and II were purified, the identity of the latter with angiotensin I-converting enzyme was established. This very fact explains the great significance of this enzyme interrelating the kallikrein-kinin system with the renin-angiotensin system. Chapter XIII summerizes the results of investigations on different kallikreins produced by exocrine glands, that is the pancreas and salivary glands. The exact functional role of these enzymes has not been established. The next chapter describes the serine proteases of the mammalian kidney and urine, also considered to be glandular kallikreins. Methods of their assay, their biochemical characteristics, origin and location, their relation to renal function and to other hormonal systems and disease are discussed. Chapter XV summerizes the interrelationships between the renal kallikrein and the regulation of blood pressure in man.

Chapter XVI describes the kallikrein-kinin system in pathological conditions and therefore contains the most data of practical use for physicians. Hereditary factors and aquired diseases relating to deficiency of proteins necessary for kinin formation are summarized in detail, and disorders leading to increased kinin formation are systematically and clearly reviewed.

The last chapter surveys the literature written in Russian. Structurally it follows the sequence of topics of the former chapters, completes them well and affords a valuable assistance to researchers unfamiliar with the Soviet literature.

The book gives a good survey of investigations up to 1977 of the kallikrein-kinin system. Though since then experimental observations have increased considerably and newer relationships have been revealed, the book is recommended to experts including physicians, biochemists, enzymologists etc., who want to keep at hand a systemic, comprehensive knowledge of the present state of this field.

I. SCHŐN

Hoppe-Seyler's Zeitschrift für Physiologische Chemie Begründet 1877 Fortgeführt von

A. Kossel, F. Knopp und K. Thomas

Vol. 361 No. 4 April 1980

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Vol. 361 No. 5 May 1980

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TONSILS

Structure, Immunology and Biochemistry

Edited by F. Antoni and M. Staub

Investigations of the Human Immune system were restricted almost entirely to the study of peripheral blood lymphocytes. Works summarized in this monograph show that human palatine tonsils represent a convenient source of lymphocytes relatively poor in phagocytic cells, offering a suitable and also sufficient material for immunological and biochemical investigations. Microenvironment and lymphocytes in the tonsils form a functional unit influencing each other throughout life. The isolated lymphocytes appear very active in the production of all types of immunoglobulins and also in the bacteriolytic enzyme, lysozyme, supporting the important role of tonsils in immunological defence.

However, the presence of activated cells characterized as "precursor" cells call attention to the role of tonsils also in lymphocyte differentiation.

The monograph can be recommended to all research workers interested in the structure, immunology and biochemistry of an important lymphoid organ, and to clinicians connected in practice with the function of tonsils.

In English — 164 pages — Cloth
ISBN 963 05 1562 8

AKADÉMIAI KIADÓ, Budapest
Publishing House of the Hungarian Academy of Sciences

ENDORPHINS '78

International Workshop Conference
Budapest, Hungary, 2-6 October 978

Ed. by L. Gráf, M. Palkovits and A. Z. Rónai

The pressure of rapid progress in research into opioid peptides forced the programme committee to select judiciously from among the leading groups. The limited number of participants at the conference held in Budapest in October 1978 acted as a means of promoting sharp and relevant discussion. The Proceedings also contains the essential points which arose during the discussions.

Comprehensive treatment was afforded to

- structural requirements of the analgesis and other activities of opioid peptides
- regulation of the biosynthesis and release of ACTH/endorphin in the pituitary and CNS
- tissue level of endogenous peptides and the choice of extraction procedure to prevent artefact formation
- microheterogeneity, origin and function of enkephalins
- different opioid receptor populations
- endogeneous opioid substances in psychiatry

In English — 335 pages — 17 × 25 cm — ISBN 963 05 1901 1 — Cloth

AKADÉMIAI KIADÓ, Budapest EXCERPTA MEDICA, Amsterdam

IMMUNOLOGY 1978

Proceedings of the Fourth European Immunology Meeting Budapest, Hungary, 12—14 April 1978

Ed. by J. GERGELY, G. A. MEDGYESI, S. R. HOLLÁN

This is the first time that the proceedings of a European Immunology Meeting are published. The volume comprises the lectures delivered at the symposia together with the invited talks of the Fourth European Immunology Meeting in Budapest, 12—14 April 1978. Up-to-date information is to be found on the most formidable problems of immunology as well as views of leading authorities on these.

From the contents: Section I — Regulation of the immune response. Section II — Ontogeny of the immune system. — Section III — Interactions between effector cells and target cells. Section IV — Immunological engineering. Section V — Steric structure and biological activity of antibodies. Section VI — Biology of the complement system. SectionVII—In vivo significance of immune complexes. Section VIII — Cell wall antigens and lipopolysaccharides. Section IX — Clinical aspects of immune phenomena.

Recommended to all concerned with theoretical and clinical immunology, haematology, biochemistry, and genetics.

In English — XVI + 532 pages — 17 × 25 cm ISBN 963 05 1878 3 — Cloth

AKADÉMIAI KIADÓ, Budapest
Publishing House of the Hungarian Academy of Sciences

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1980, III. 21. — Terjedelem: 8,75 (A/5) ív, 30 ábra

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20. XI. 1980 HU ISSN 0001—5253 Index: 26.030

VOLUME 15
NUMBER 4
1980

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

ABBPAP 15 (4) 259–346 (1980)

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

STRAUB F. BRUNÓ és ERNST JENŐ

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (4), pp. 259–261 (1980)

Quantitative Determination of Thromboxane Synthesis in Blood Samples by Video-densitometry

(Short communication)

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(Received May 16, 1980)

Hamberg and coworkers (1975) have reported that human thrombocytes synthesize an unstable intermediate TxA_2 during the conversion of arachidonic acid into the stable metabolite TxB_2 through the two prostaglandin endoperoxides PGG_2 and PGH_2.

TxA_2 is responsible for irreversible platelet aggregation (Hamberg et al., 1975) and was shown to induce contraction of smooth muscle (Svensson et al., 1975) and rabbit aorta.

Identification and quantitative determination of thromboxanes are essential for the understanding of their physiological role and can be accomplished by utilizing a combined gas chromatograph–mass spectrometer or, in biological assay systems, by determining the rate of inhibition of platelet aggregation (Ho et al., 1976a, b).

This report describes a rapid method for determining the rate of TxB_2 synthesis in blood samples using TLC and video-densitometry.

30–50 ml of blood was drown from each of the volunteers into plastic test tubes containing 1/10 volume of anticoagulant solution (3.8% w/v sodium citrate-5.5 H_2O). None of the patients had received aspirin or any other non-steroidal anti-inflammantory drug known to inhibit thromboxane biosynthesis for at least ten days prior to the study. Blood samples were centrifuged at 500 *g* for 8 min at room temprerature to remove red blood cells. Plasma containing the thrombocytes was decanted and centrifuged at 3000 *g* for 15 min at room temperature to obtain the platelet fraction. This platelet pellet was washed twice with 10 ml of 0.9% NaCl solution and centrifuged under the above conditions. The washed platelet fraction was resuspended in 10 ml of 15.4 mM Tris-HCl buffer, pH 7.4, containing 136 mM NaCl and L-tryptophan (2 mg/10 ml). To this suspension 20 μl of arachidonic acid (10 mg/ml) in ethanol was added. The mixture was incubated at 37 °C for 30 min, and then diluted with 15 ml of water and adjusted to pH 3 with 1.0 ml of 2 M citric acid. The acid-soluble lipids were extracted with 50 ml of diethyl ether and acids were removed by extraction with 30 ml of

Abbrevations: PG, prostaglandin; Tx, thromboxane.

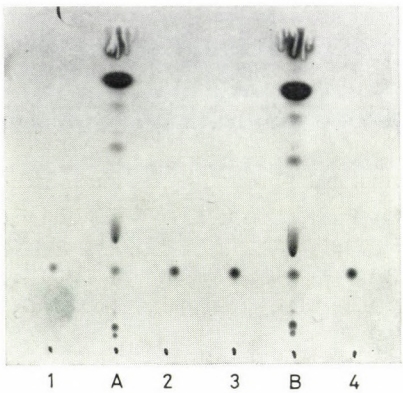

Fig. 1. Thin layer chromatogram of TxB_2 synthesized by human platelets from the precursor arachidonic acid. Different amounts of standard TxB_2 solution were also applied onto the plate. For more details see text. 1: 1 μg TxB_2; 2: 2 μg TxB_2; 3: 3 μg TxB_2; 4: 4μg TxB_2; A: 10 μl sample; B: 20 μl sample

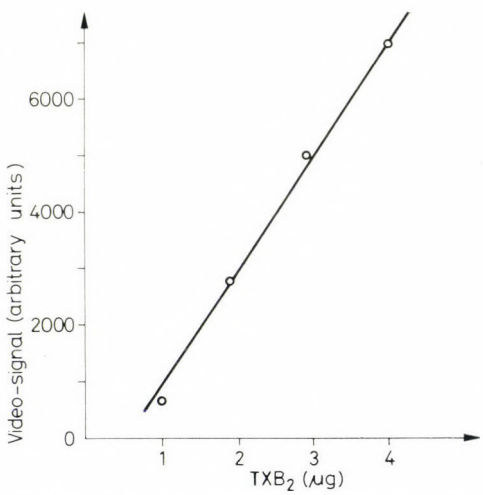

Fig. 2. Calibration curve obtained by video-densitometry for determining the amount of TxB_2 applied onto the silica gel-coated TLC plate

water two times. The ethereal phase was evaporated to dryness below 50 °C, and the residue was taken up in 0.1 ml of ethanol.

For the quantitative determination of TxB_2 in these samples, TxB_2 was separated from all other lipids on TLC plates. For this purpose Merck DC-Plastikfolien Kieselgel 60 plates (20 cm × 20 cm × 0.2 mm) were used. Different concentrations of the reference TxB_2 solution were applied onto the plates (1, 2, 3 and 4 μg) 3 cm apart, and the sample solution was also applied in at least two differ-

ent concentrations. The plate was developed in the organic solvent chloroform–methanol–glacial acetic acid, 90 : 5 : 5 at room temperature. After drying the plates were developed by the method of Touchstone et al. (1978). Under these conditions TxB_2 had an R_f value of 0.29. Figure 1 shows a chromatogram suitable to determine the TxB_2 concentration of the applied sample, using known amounts of TxB_2 as a reference. The quantitative determination of TxB_2 on TLC plates was performed by video-densitometry (Telechrom S, Chinoin, Nagytétény) as described by Dévényi (1976) and Pongor et al. (1978) Figure 2 shows the calibration curve obtained by video-densitometry. The video signals of TxB_2 were plotted against their concentrations and the calibration curve was used to determine the amount of TxB_2 synthesized by the platelet fraction.

We determined thromboxane synthesis in several human platelet preparations and found it to vary between 28.5–46.1 μg $TxB_2/10^{10}$ thrombocytes/hour.

So far, only one clinical investigation has been reported in which ADP stimulated TxB_2 synthesis of normal and diabetic platelets was compared (Ziboh et al., 1979). In this study the radioisotope technique was used and the results were expressed as percentage of ^{14}C recovered in TxB_2 per mg of homogenized platelet proteins. Therefore, these values cannot be compared with ours.

The authors express their thanks to Ms J. Kramer for performing and evaluating the video-densitometric measurements. We also thank Ms J. Szekeres and Ms Gy. Botos for technical help.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (4), pp. 263—273 (1980)

Specificity of Pancreatic Elastase with Tripeptidyl-*p*-Nitroanilide Substrates

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(Received March 28, 1980)

1. The kinetic properties of pancreatic elastase (E.C. 3.4.21.11) were investigated with 33 tripeptidyl-*p*-nitroanilide substrates, and the K_m, k_{cat} and k_{cat}/K_m values were determined.

2. The individual contributions of the substrate side chains to the kinetic constants were evaluated by regression analysis. As a result of the additivity of the contributions, the kinetic parameters of any substrate constructed from the amino acid investigated can be predicted.

3. Suc-D-Phe-Pro-Ala-*p*NA was the best substrate, as far as the K_m (Michaelis constant) is concerned. The compound was synthesized and assayed. An excellent correlation was observed between its calculated (14 μM) and experimentally determined (15 μM) K_m values. The aldehyde derivative of this substrate is a competitive inhibitor of elastase ($K_i = 0.6$ mM).

4. The contribution values of the best substrates permitted us to characterize the topography of subsites involved in the formation of the enzyme-substrate complex. This, in turn, led us to the conclusion that the S_3-P_3 interaction is predominant and rate-limiting, while the S_1-P_1 interaction is relatively less important in the binding of good substrates.

Introduction

Pancreatic elastase (E.C. 3.4.21.11) is an endopeptidase which splits the peptide bond at the appropriate rate, near small non-polar amino acids (Narayanan, Anwar, 1969; Kaplan, Dugas, 1969) of which alanine is given preference at the P_1 position (notation by Schechter and Berger, 1967).

The kinetic properties of the enzyme have usually been investigated, besides its natural substrate, elastin, with amino acid and peptide esters (Bender et al., 1966; Bender, Marshall, 1968; Kaplan, Dugas, 1969; Gertler, Hofman, 1970) and with chromogenic substrates (Bieth et al., 1974; Kasafirek et al., 1974; Zimmerman, Ashe, 1977).

In earlier studies substrates of monotonous or slightly varied amino acid sequences, mainly combinations of Ala and Pro, have been tested (Janoff, Basch, 1971; Atlas, Berger, 1972; Atlas, 1974; Bieth et al., 1974; Feinstein, Janoff, 1975;

Abbreviations: Bz–, benzoyl-; Z–, benzyloxycarbonyl-; Suc–, succinyl-; -*p*NA, *p*-nitroanilide; Nle, norleucine; Pip, pipecolic acid.

Powers et al., 1975; Zimmerman, Ashe, 1977). In the present investigations substrates having a more diverse amino acid sequence (e.g. seven various residues at the P_2 and eleven at the P_3 position) were assayed to obtain information about the topography of the binding site.

The specificity can be ascribed to the tertiary structure determined by X-ray crystallography (Shotton, Watson, 1970) i.e. to the structure of the hydrophobic crevice of the enzyme (Blackburn, 1976). More information has been obtained about further segments of the binding site with peptidyl–chloromethyl ketone inhibitors (Tuhy, Powers, 1975; Powers et al., 1975).

Using peptide substrates of different chain length Atlas (1974) has found that the enzyme binds most effectively substrates which are composed of at least eight amino acid residues, and the peptide bond is cleaved between the fifth and sixth residues.

It has been found in previous studies with subtilisin and trypsin (Pozsgay et al., 1978; 1979) that the regression analysis of kinetic parameters can be successfully applied to characterize the role of individual amino acid side chains in the interaction of serine proteases with their substrates. In the present studies the structure–function relationship of tripeptidyl-*p*-nitroanilide substrates was investigated with pancreatic elastase. The results obtained permitted us to suggest a probable construction for a part of the substrate binding site in the enzyme.

Materials and methods

Chemicals

Porcine pancreatic elastase, twice recrystallized (Lot No. 117C-9001) was obtained from Sigma Chem. Co. (St. Louis, Mo., USA). The enzyme was stored in aqueous suspension at 4 °C.

Substrates No. 1, 3, and 7 and Suc-D-Phe-Pro-Alaaldehyde were generously supplied by Dr S. Bajusz (Institute for Drug Research, Budapest, Hungary).

Substrates No. 4, 6, 9, 11, 12, 13, 14, 16, 17, 18, 21, 23, 25, 29 and 31 were synthesized in our laboratory. The homogeneity of the products was analyzed by thin-layer adsorption chromatography (Kieselgel G; elution mixture : ethyl acetate : pyridine : acetic acid : water = 240 – 60 : 20 : 6 : 11), their composition was then controlled by elementary analysis, and only pure products were assayed further.

All the other substrates were generously supplied by Mr. R. Simonsson (KABI Peptide Research, Mölndal, Sweden).

All the reagents used for synthesis and for kinetic measurements were the best commercially available products.

Enzyme activity

Enzyme activity was determined at 37 °C in 50 mM Tris-HCl buffer, pH 8.1. The assays were carried out in 1 cm cuvettes in a Unicam SP-500 spectrophotometer equipped with a thermostated cell holder and a recorder. The liberation of *p*NA from the substrate was followed for 3 – 5 min by measuring the increase

Table 1

Kinetic parameters of elastase

Measured with tripeptidyl-*p*-nitroanilide substrates in 50 mM Tris-HCl buffer, pH 8.1, at 37 °C

		Substrate subsites				$1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1}s^{-1}$
		P_4	P_3	P_2	P_1			
A.	1.	Z	D-Phe	Pro	Arg	57.10	0.00144	82.10
	2.	Z	D-Phe	Gly	Arg	46.10	0.00304	140.00
	3.	Z	Phe	Pro	Arg	30.00	0.00235	70.60
	4.	Z	Arg	Val	Leu	14.50	0.23200	3360.00
	5.	Bz	Asn	Pro	Arg	11.60	0.00294	34.20
	6.	Suc	Ala	Ala	Ala	11.00	0.37600	4140.00
	7.	Z	Phe	Val	Arg	10.00	0.00465	46.59
	8.	Bz	Phe	Val	Arg	7.30	0.00136	9.33
	9.	Bz	Arg	Val	Leu	6.29	0.09420	592.50
	10.	Bz	Ile	Pro	Arg	6.10	0.00256	15.60
	11.	Bz	Arg	Nle	Nle	5.49	0.18830	1034.70
	12.	Suc	Gly	Val	Ala	5.49	0.01770	97.10
	13.	Bz	Phe	Gly	Arg	5.21	0.00260	13.50
	14.	Z	Arg	Nle	Nle	5.00	0.15700	785.00
	15.	Bz	Ile	Val	Arg	2.05	0.00272	5.57
	16.	Suc	Gly	Val	Nle	1.35	0.00196	2.65
	17.	Suc	Gly	Ala	Leu	1.10	0.03210	35.30
	18.	Suc	Gly	Val	Val	1.10	0.00804	8.84
B.	19.		D-Phe	Pro	Ala	50.00	0.02940	1470.00
	20.		D-Phe	Pro	Arg	28.00	0.00110	32.20
	21.		Ala	Ala	Ala	21.00	0.11780	2473.00
	22.		D-Lys	Pro	Arg	13.50	0.00086	11.70
	23.		Gly	Val	Ala	11.00	0.08834	971.70
	24.		D-Arg	Pro	Arg	10.00	0.00144	14.42
	25.		Arg	Val	Leu	9.71	0.01070	103.90
	26.		D-Val	Pro	Arg	7.19	0.00193	13.90
	27.		D-Val	Pip	Arg	7.10	0.00158	11.40
	28.		D-Ile	Pro	Arg	6.29	0.00185	11.70
	29.		Gly	Ala	Leu	6.00	0.05890	352.60
	30.		D-Phe	Pip	Arg	5.65	0.00156	8.81
	31.		Arg	Pro	Arg	2.30	0.00098	2.26
	32.		D-Phe	Val	Arg	1.60	0.00067	1.07
	33.		D-Phe	Phe	Arg	0.40	0.00352	1.41

in absorption at 405 nm. The molar absorption coefficient of *p*NA was taken as 10 600 (AB Bofors, Data sheet, 1974). The final volume of the reaction mixture was 1.5 ml. The concentrations of the substrates ranged from 7 to 1000 μM enzyme concentration was 0.3 – 14 μM.

Regression analysis

For the regression analysis of the kinetic constants the method suggested by Free and Wilson (1964) was used as described earlier (Pozsgay et al., 1979).

Results

Determination of the rate of hydrolysis

During 3 – 5 min of hydrolysis the rate of *p*-nitroaniline liberation was directly proportional to time. The enzyme assays were carried out in aqueous

Table 2

Calculated contribution of the subsites of tripeptidyl-p-nitroanilide substrates to the kinetic parameters (Series A from Table 1)

Subsite	Residue	Contribution to		
		$1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1}s^{-1}$
P_1	Ala	5.36	0.0223	318.2
	Nle	1.22	0.0065	223.8
	Val	0.97	0.0126	229.9
	Arg	− 1.19	−0.0073	− 113.9
	Leu	− 1.53	−0.0038	− 170.8
P_2	Pro	9.89	−0.0097	9.3
	Ala	0.66	0.0362	537.9
	Val	− 1.84	−0.0043	110.7
	Gly	− 3.34	0.0054	351.0
	Nle	− 9.74	−0.0001	−1350.0
P_3	D-Phe	36.48	−0.1072	−1356.0
	Arg	3.88	0.0680	1155.0
	Phe	3.78	0.0900	− 936.8
	Asn	− 2.99	−0.0153	684.3
	Ile	− 7.76	−0.0822	− 487.5
	Ala	−12.13	0.3683	3821.0
	Gly	−18.24	0.0285	− 423.2
P_4	Suc	6.36	−0.1040	−1289.0
	Z	1.97	0.0594	1064.0
	Bz	− 6.23	0.0234	8.8
Overall contribution	(μ)	12.60	0.0628	581.9

Table 3

Calculated contribution of the subsites of tripeptidyl-p-nitroanilide substrates to the kinetic parameters (Series B from Table 1)

Subsite	Residue	Contribution to		
		$1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1}s^{-1}$
P_1	Ala	18.90	0.0211	1138.0
	Leu	18.29	0.0033	−182.0
	Arg	− 9.28	−0.0070	−305.0
P_2	Pro	10.67	0.0016	− 80.8
	Pip	− 0.55	0.0016	− 93.7
	Phe	− 7.16	−0.0063	−106.7
	Val	−10.17	0.0010	−106.6
	Ala	−15.85	0.0017	589.7
P_3	D-Phe	9.06	−0.0147	48.0
	Ala	6.58	0.0860	380.6
	D-Lys	0.13	−0.0151	32.7
	D-Val	− 0.62	−0.0143	40.2
	D-Arg	− 3.38	−0.0146	− 0.4
	D-Ile	− 7.08	−0.0141	32.8
	Gly	− 9.10	0.0449	−424.4
	Arg	−11.07	−0.0150	23.3
Overall contribution	(μ)	11.98	0.0214	365.3

medium, because the presence of solvent (e.g. dimethyl sulfoxide, acetone, ethanol, pyridine, etc.) reduced the rate of reaction markedly. As shown by thin-layer adsorption chromatography, only the P_1-P_1' linkage was in fact split during the reaction time.

The experimentally determined kinetic constants of tripeptidyl-*p*-nitroanilide substrates are summarized in Table 1. On practical consideration, the $1/K_m$ values are presented, because in regression analysis the reciprocal value of the Michaelis constant is used, as the method necessitates higher constants for the better substrate than for the poorer one.

Regression analysis of the kinetic constants

The influence of the amino acid sequence on the binding of substrates and the rate of hydrolysis can be characterized quantitatively by the method of regression analysis introduced by Free and Wilson (1964) for testing the biological activity of drugs. This mathematical method reduces the kinetic constants to additive components, the so-called "contributions", which indicate the share of

an amino acid side chain in the kinetic constant at a given subsite (Tables 2 and 3).

Owing to the additivity of the contributions, from the first series of data (Table 2) the kinetic parameters of 525, and from the second (Table 3) those of 120 substrates with various sequence combinations can readily be obtained. The signs of the contributions indicate the direction in which the kinetic parameter of the substrate is shifted by the side chain at a given subsite, compared to μ, the overall contribution, characteristic of the series studied. The additivity of the computed contributions is evidenced by the values of the correlation coefficients (r, around 0.9) obtained by comparing the appropriate calculated and measured kinetic constants (Table 4).

From the residues that have the highest values of contribution to the kinetic constants at given subsites, new tripeptides can be derived, which can be considered as the "best" substrates for the enzyme, as far as their $1/K_m$, k_{cat} or k_{cat}/K_m

Table 4

Correlation between kinetic constants measured and calculated from the contributions

Kinetic parameter	Correlation coefficient (r)	
	Series A	Series B
$1/K_m$	0.9860	0.9684
k_{cat}	0.9723	0.9043
k_{cat}/K_m	0.8608	0.9999

Table 5

Amino acid sequence and kinetic constants of calculated optimal substrates

Substrate	Kinetic parameter		
		Calculated	Measured
Series A			
Suc-D-Phe-Pro-Ala-*p*NA	$1/K_m$, mM^{-1}	70.6	65.3
Z-Ala-Ala-Ala-*p*NA	k_{cat}, s^{-1}	0.549	—*
Suc-Ala-Ala-Ala-*p*NA	k_{cat}, s^{-1}	0.385	0.376
Z-Ala-Ala-Ala-*p*NA	k_{cat}/K_m, $M^{-1}s^{-1}$	6324.0	—*
Suc-Ala-Ala-Ala-*p*NA	k_{cat}/K_m, $M^{-1}s^{-1}$	3970.0	4140.0
Series B			
D-Phe-Pro-Ala-*p*NA	$1/K_m$, mM^{-1}	50.6	50.0
Ala-Ala-Ala-*p*NA	k_{cat}, s^{-1}	0.130	0.118
Ala-Ala-Ala-*p*NA	k_{cat}/K_m, $M^{-1}s^{-1}$	2474.0	2473.0

* Insoluble in the absence of organic solvent

values are concerned. Z-Ala-Ala-Ala-*p*NA, the "best" substrate predicted for the k_{cat} and k_{cat}/K_m values, is non-polar, and only its succinyl derivative can be used for kinetic measurements. Because of the strongly negative contribution of a P_4 Suc residue, weaker kinetic parameters are expected than in the case of the derivative containing Z as the P_4 residue. The predicted tripeptidyl-*p*-nitroanilides were synthesized and assayed kinetically (Table 5). The measured values of the kinetic parameters correlate nicely with the values obtained by calculations.

Effect of a tripeptidyl inhibitor

Suc-D-Phe-Pro-Ala-*p*NA, the substrate with the best binding constant is characterized by small K_m (15 μM) and small k_{cat} (0.002 s^{-1}) values. Accordingly, it possesses a good binding and a poor hydrolyzing capacity. Therefore, the analogue of the substrate having the best binding capacity can be expected to be an effective inhibitor for the enzyme. To test this assumption, the aldehyde derivative of this compound, Suc-D-Phe-Pro-Ala aldehyde, was used as inhibitor with Suc-Ala-Ala-Ala-*p*NA substrate (conc.: $60-500$ μM). The type of inhibition was competitive, as indicated by the Lineweaver–Burk plot. By plotting the data according to Dixon (1953) an inhibitory constant of 0.6 mM was obtained. Thus, the aldehyde derivative of the substrate which has the most favourable $1/K_m$ value is a competitive inhibitor of the enzyme.

Discussion

The catalytic efficiency of the serine proteases is influenced by the subsite determining the primary specificity as well as by the length and sequence of the peptide substrate.

The catalytic and the proteolytic constants found with various peptidyl substrates are characterized by small values (see Table 1). This may be explained by the fact that our substrates are tripeptides, whereas according to Atlas (1974) the binding site of elastase is relatively large. To obtain the optimum hydrolysis rate an octapeptide is required. On the other hand, the $S_1'-P_1'$ interaction, which can elevate the reaction rate markedly, cannot either be formed between the enzyme and the rapidly liberated *p*NA residue of the substrate.

The contributions of amino acid side chains (Tables 2 and 3) represent individual and quantitative characteristics. This allowed us to treat the amino acid side chains within each set of substrates separately, which, in turn, permitted us to draw conclusions concerning the enzyme–substrate interaction at each binding site.

S_4-P_4 interaction

The kinetic constants indicate that the presence of a P_4 residue improves the catalytic action of elastase (see Tables 1 and 5). The extension of substrates by protecting groups at the N-terminus improves both the $1/K_m$ and the k_{cat}/K_m

values (compare, e.g. Nos 6 and 21, Nos 9 and 25, or Nos 4 and 25 analogous substrate pairs).

The Suc residue increases the $1/K_m$ value of the substrate, which suggests an affinity higher than that found with substrates containing a Z or a Bz group at the P_4 position. This correlates with the observation of Feinstein et al. (1973) and Kasafirek et al. (1974, 1976), that the succinyl group and other dicarbonic acid protective groups are more favourable for binding than the hydrophobic side chains. In contrast, the rate of hydrolysis, i.e. both k_{cat} and k_{cat}/K_m, are increased by aromatic protective groups.

S_3-P_3 interaction

It can be seen in Table 2 that, as regards $1/K_m$, aromatic amino acid residues as well as residues with large steric requirement (D-Phe, Phe, Arg) ensure a strong binding. Amino acids having D configuration are especially favourable for binding. $1/K_m$ is decreased by small non-polar amino acid substituents.

For the efficiency of hydrolysis small hydrophobic amino acids, particularly Ala or Gly, are required. However, the rate of hydrolysis is slower with long side chains.

S_2-P_2 interaction

Hydrophobic side chains are preferable at the P_2 position to obtain a reasonable affinity and hydrolysis rate.

Binding is particularly good with Pro, although, Ala, Pip or Val are also acceptable at this position. Pro has been found by Tuhy, Powers (1975) 4–5 times more effective than Ala in peptide-chloromethyl ketones, while Phe has been proved to be substantially less fit for binding. Our studies with other serine proteases, i.e. with thrombin and trypsin, have also indicated that the contribution of a P_2 Pro residue to $1/K_m$ exceeds that of other residues at the same position. The distinct preference for Pro at the P_2 position seems to be a general feature of serine proteases (Pozsgay et al., 1978; 1981a; 1981b).

The rate of hydrolysis is enhanced by small non-polar amino acids, such as Ala > Gly > Val, whereas the catalytic activity is diminished by side chains with large steric requirement.

Primary specificity of elastase, the S_1-P_1 interaction

In agreement with observations in other laboratories (Schechter, Berger, 1968; Narayanan, Anwar, 1969; Kaplan, Dugas, 1969; Gertler, Hofman, 1970; Kaplan et al., 1970; Gold, Shalitin, 1975; Atlas, Berger, 1973; Zimmerman, Ashe, 1977), Ala was found to be the best substituent at the P_1 position both for an efficient binding and for a favourable hydrolysis rate, but other aliphatic side

chains, i.e. Leu, Nle or Val, were also acceptable. The polar Arg with its large steric requirement and a positive charge, however, elicites weak binding and slow hydrolysis.

Structure of the binding site of pancreatic elastase

A comparison of the best $1/K_m$, k_{cat} and k_{cat}/K_m values presented in Tables 2 and 3 reveals that preference is not always given to the same side chain at a given subsite (see in addition the best sequences in Table 5). This assumption is manifested in the fact that Suc-D-Phe-Pro-Ala-*p*NA is a poor substrate (small k_{cat}), although, it has good binding properties. If the values obtained for $1/K_m$ are considered to be proportional to the degree of binding, the properties of the substrate binding site can be inferred from these data, and the S_1-S_4 binding sites of the enzyme can be characterized as follows.

The S_1 and S_2 subsites are small hydrophobic crevices. The S_3 site can accomodate a non-polar or an aromatic side chain, which requires a relatively large cavity. At the S_4 subsite probably there is a polar side chain which forms an ionic interaction with the Suc residue. This pocket is most probably not a large one.

In this model only the steric requirements and the polarity of the residues were taken into account. Other characteristics, e.g. hydrophobicity, etc., should also be examined to obtain a more realistic picture.

The topography suggested here can be compared with the X-ray structure of elastase (Shotton, Watson, 1970). The hydrophobic substrate pocket is built up from Val-216, Thr-226 and Ser-188, and S_1 Val-216 may form a hydrophobic interaction with the hydrocarbon (methyl) side chain at the P_1 subsite. The hydrogen bridge formed by Pro and Gly-193 can be considered as the S_2-P_2 interaction (at the same time the crevice is hydrophobic). The carbon chain of Gln-150 may form a strong hydrophobic interaction with D-Phe (S_3-P_3), as suggested by Atlas (1974). Finally, the free carboxyl group of the Suc residue may form a hydrogen bond with the NH group of the backbone at the S_4 site (S_4-P_4).

Relative importance of the subsites

The data presented in Table 6 reveal that the P_3 amino acid side chains represent 50–70 per cent of the contribution to the kinetic parameters when the best substrates are taken. Surprisingly, the contribution of P_1, which determines the primary specificity of the enzyme, amounts only to about 5–7 per cent. Hence, we concluded that the enzyme–substrate interaction at the P_3 position is of great importance. This may be explained by the following.

The rate-limiting step in the function of proteolytic enzymes is the decomposition of the acyl–enzyme complex (Bender, Marshall, 1968). If the substrate is a peptide or a protein, it forms interactions with the enzyme at more than one subsite. Since the contribution of individual in the residue substrate to $1/K_m$ varies at different subsites (Table 6), we may assume that the formation or the

Table 6

Relative significance of the subsites of optimal substrates, as calculated from the contributions in Table 2

Subsite	$1/K_m$, mM^{-1}			k_{cat}, s^{-1}			k_{cat}/K_m, $M^{-1}s^{-1}$		
	Sequence	Contribution	%*	Sequence	Contribution	%*	Sequence	Contribution	%*
P_4	Suc	6.3	10.8	Z	0.059	12.2	Z	1064	18.5
P_3	D-Phe	36.5	62.9	Ala	0.268	75.8	Ala	3822	66.6
P_2	Pro	9.9	17.0	Ala	0.036	7.4	Ala	538	9.4
P_1	Ala	5.4	9.3	Ala	0.022	4.0	Ala	318	5.5

* The overall contribution (μ) was neglected in the calculations

cleavage of bonds does not proceed in one step only, but according to the number of segments (subsites). It seems reasonable to suppose that the rate of each step (the formation and/or break of subsite interactions) is inversely proportional to the strength of the bond formed. Thus, the rate of hydrolysis is determined by the slowest step, i.e. by the breaking of the strongest bond in the acyl–enzyme complex.

The role of the S_3-P_3 interaction is predominant in the binding of substrates, because it produces the largest contribution. The substitution of Ala for D-Phe at the P_3 position in Suc-D-Phe-Pro-Ala-*p*NA reduces the value of $1/K_m$ to one third of the original (from 65.3 mM^{-1} to 22.08 mM^{-1}, calculated), while it results in a 150-fold increase in the value of k_{cat} (from 0.0024 s^{-1} to 0.3397 s^{-1}). Hence, the weakening of the stability of the acyl–enzyme complex promotes, to a certain extent, a faster release of the product. It is to be pointed out that even in a monotonous sequence, such as Z-Ala-Ala-Ala-*p*NA, there are great differences between the values of contribution, depending on the position of Ala (Table 6).

We should like to emphasize that our previous observation with other serine proteases, e.g. with trypsin and thrombine, also indicated that the P_3 residue may be of special importance, and the relative measure of the contribution of the subsites is practically similar to that found with elastase (Pozsgay et al., 1979; 1981a, b).

The excellent technical assistance of Miss S. Merész is gratefully acknowledged.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (4), pp. 275–285 (1980)

Purification and some Properties of Polynucleotide Kinase from Rat Liver Nuclei

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(Received March 26, 1980)

Polynucleotide kinase was purified from crude extracts of rat liver nuclei by affinity chromatography on DNA agarose. At optimal pH (5.5) and at saturating concentrations of ATP and DNA, the purified enzyme was found to express maximal activity in the presence of 0.10–0.15 M NaCl; higher salt concentrations inhibited the activity. At the optimal pH and NaCl concentration, the apparent K_M for 5'-OH-DNA at 100 μM ATP was 46.2 μM and the apparent K_M for ATP at 1 mM 5'-OH-DNA was 15.8 μM. Polynucleotide kinase was protected against heat inactivation by ATP as well as by 5'-OH-DNA at low and moderately high NaCl concentrations, which suggests that under these conditions the enzyme reacts according to a random reaction mechanism. Studies on the heat inactivation of the enzyme in the presence of 5'-OH- or 5'-P-DNA revealed that protection occurs only if 5'-OH-DNA is present, at NaCl concentrations permitting the enzyme to bind DNA.

Introduction

Polynucleotide kinases (polynucleotide 5'-hydroxyl-kinase, EC 2.7.1.78) catalyze the transfer of the terminal phosphate group of ATP to the 5'-OH-termini of polynucleotides, oligo- and mononucleotides. Such enzymes were isolated from *Escherichia coli* infected by T4 (Richardson, 1965) and T2 phages (Novogrodsky, Hurwitz, 1965, 1966; Novogrodsky et al., 1966), from rat liver nuclei (Novogrodsky et al., 1966; Ichimura, Tsukada, 1971; Teraoka et al., 1975; Levin, Zimmermann, 1976; Pheiffer, Zimmermann, 1979), from the nuclei of Landschütz carcinoma and BHK fibroblast cell lines (Evans et al., 1975), from calf thymus (Austin et al., 1978), and identified in human lymphocytes (Pedrali Noy et al., 1974).

The substrate specifity (Richardson, 1965; van de Sande, Bilsker, 1973; Narang et al., 1975; Lillehaug, Kleppe, 1975a; Lillehaug et al., 1976) and reaction mechanism (Lillehaug, Kleppe, 1975a, b; Lillehaug et al., 1976; Sano, 1976) of T4-induced polynucleotide kinase have been studied in detail; however, relatively

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Abbreviations used: DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; K_M, Michaelis constant; EDTA, ethylene-diamino-tetra-acetic acid.

little is known about polynucleotide kinases from different mammalian sources. As mammalian polynucleotide kinases are supposed to play an important role in the synthetic and repair processes of DNA (see e.g. Levin, Zimmermann, 1976), it was of interest to further investigate the properties of the rat liver enzyme in view of future functional studies, and also to further compare the enzyme with the well-known T4-induced polynucleotide kinase.

In the present work we studied the dependence of polynucleotide kinase activity upon ionic strength and carried out a detailed investigation of the heat inactivation of the enzyme, in order to draw conclusions as to the possible reaction mechanism and to characterize the bonds between the enzyme and DNA.

Materials and methods

Alkaline phosphatase from *Escherichia coli* 3747 (EC 3.1.3.1) and porcine muscle glyceraldehyde-3-phosphate-dehydrogenase (EC 1.2.1.12), purified according to Torriani (1966) and Elődi and Szörényi (1956), respectively, were the gifts of Dr Judit Nagy and Dr Magda Solti from this Institute. Phosphoglycerate kinase (EC 2.7.2.3) and pancreatic DNase I (EC 3.1.4.5) were Sigma (St. Louis, Missouri, USA) preparations.

ATP and high molecular weight DNA from chicken erythrocytes were from Reanal (Budapest, Hungary). Sucrose and 2-mercaptoethanol were Merck products (Darmstadt, Germany), bovine serum albumin, 2-deoxy-D-ribose and 3-phosphoglycerate were purchased from Sigma (St. Louis, Missouri, USA) and cysteine from Fluka (Buchs, Switzerland). All the other chemicals used were the analytical grade products of Reanal (Budapest, Hungary).

[γ-^{32}P]-ATP (specific activity 10–50 mCi/mmole) was prepared by the method of Glynn and Chappell (1964).

5'-OH-DNA was prepared from high molecular weight DNA from chicken erythrocytes. DNA (2.5 mg/ml) was digested with pancreatic DNase I (20 μg/ml) in the presence of 20 mM sodium acetate buffer (pH 5.2) and 1 mM $MgCl_2$ at 37 °C for 20 min, precipitated with 10% trichloroacetic acid, redissolved in 10 mM EDTA and precipitated three times with absolute ethanol. DNA was then dissolved in 24 mM Tris-HCl buffer (pH 8.2) and 5'-termini were dephosphorylated by incubation at 65 °C for 30 min with *E. coli* alkaline phosphatase added in a ratio of 0.2 U/μmole DNA nucleotide. (The activity of alkaline phosphatase was determined according to Garen and Levinthal (1960) with *p*-nitrophenyl-phosphate as substrate; one enzyme unit is defined as the amount of enzyme which catalyzes the formation of 1 μmole of *p*-nitrophenol in 1 min, at pH 8.0, 25 °C.) The reaction mixture was then treated with one volume of phenol, and after centrifugation DNA was precipitated from the aqueous phase by ethanol. The precipitate was dissolved in 1 mM Tris-HCl (pH 7.0) and extensively dialyzed against the same solution. The 5'-OH-DNA prepared in this way is practically denatured (owing to incubation at 65 °C and precipitation by trichloroacetic acid) and does not show any hyperhcromicity.

Purification of polynucleotide kinase

Preparation of the crude extract. Crude extracts were prepared from the livers of inbred PVG/c rats of both sexes and subjected to pH 5 treatment essentially as described by Ichimura and Tsukada (1971), except that the isolated nuclei were disrupted by sonication for 3×30 sec in an MSE sonicator at a power of 150 W.

DNA agarose chromatography. Polynucleotide kinase was further purified by affinity chromatography on DNA agarose according to the method described by Schaller et al. (1972). A 0.9 $cm^2 \times 10$ cm column was prepared of DNA agarose containing 4% agarose and 1% high molecular weight DNA from chicken erythrocytes. (It must be noted that the commercial chicken erythrocyte DNA preparation contains many 5'-OH termini and is a good substrate for polynucleotide kinase.) The column was extensively washed with buffer A (0.01 M Tris-HCl, pH 7.5 + 1 mM EDTA + 5 mM 2-mercaptoethanol + 0.1 M NaCl). Throughout the whole procedure the flow rate of the column was 14 ml/hour. The crude extract was loaded on the column and recirculated once, and the column was then washed with buffer A until the eluate was protein-free. Polynucleotide kinase was eluted with a linear gradient (2×25 ml) of buffer A containing 0.1 – 0.6 M NaCl. The elution of the enzyme started at 0.22 – 0.24 M NaCl. The gradient fractions were assayed for polynucleotide kinase activity; the active fractions were pooled, concentrated and in some cases diafiltrated in an Amicon Model 52 or Model 8MC cell using a PM-10 membrane.

Polynucleotide kinase assay

Kinase activity was measured essentially as described by Ichimura and Tsukada (1971) with minor modifications. Reactions mixtures (0.3 ml), unless otherwise specified, contained 60 mM sodium acetate buffer (pH 5.5), 10 mM $MgCl_2$, 16 mM 2-mercaptoethanol, 100 μM [γ-^{32}P]-ATP, 1 mM 5'-OH-DNA nucleotide and a rate-limiting amount of enzyme. The reaction was usually started by the addition of the enzyme after a 2-min. preincubation of the rest of the components at 37 °C. After incubation at 37 °C, the reaction was stopped by the addition of 0.1 ml of 0.5 M NaOH followed by incubation at 100 °C for 20 min. The tubes were chilled in ice, and after the addition of 0.2 ml of 50 mM KH_2PO_4 + + 50 mM sodium pyrophosphate, DNA was precipitated by 3 ml of 10% trichloroacetic acid, collected on Sartorius nitrocellulose membrane filters (pore size 0.45 μ, previously soaked in 0.5 M KH_2PO_4 for at least 4 hours) and washed with 4×5 ml of ice-cold trichloroacetic acid. Radioactivity was determined in distilled water by Cerenkov radiation in a Packard Tricarb scintillation spectrometer. Specific activities are expressed as nmoles of phosphate incorporated into DNA (i.e. rendered acid-insoluble) by 1 mg of protein in 10 minutes.

ATPase assay

ATPase activity of the enzyme purified on DNA agarose was tested in a reaction mixture identical with that used for the polynucleotide kinase assay, except that 5'-OH-DNA was omitted. The reaction was stopped by the addition of 0.3 ml of 10% trichloroacetic acid and the precipitate was removed by centrifugation. $^{32}P_i$ was determined in the supernatant by the filter precipitation method of Reimann and Umfleet (1978).

Analytical procedures

DNA concentration was determined by the diphenylamine test (Burton, 1956) with 2-deoxy-D-ribose as standard.

DNA concentrations are expressed as μmoles of nucleotide per liter solution.

Protein concentration was measured by the Folin reaction (Lowry et al., 1951) with bovine serum albumin as standard. In the case of protein solution containing Tris, 2-mercaptoethanol, glycerol or sucrose, aliquots were precipitated by trichloroacetic acid, centrifuged and the pellets dissolved in 0.15 M NaCl before the Folin assay.

The NaCl concentration in gradient fractions was calculated from conductivity measurements using an appropriate calibration curve.

Results and discussion

Owing to contaminating enzyme activities (nuclease, ATPase) interfering with the polynucleotide kinase assay, enzyme activity could not be measured at earlier stages of purification (i.e. in the liver homogenate or before the pH 5 treatment). Therefore, the degree of purification achieved in these steps could not be calculated.

The specific activity of the crude extract ranged from 0.1 to 0.3 nmoles of P/mg protein/10 min, which is about one order of magnitude lower than the values reported by Teraoka et al. (1975) (3.3 nmoles of P/mg/10 min) and by Levin and Zimmermann (1976) (1.7 nmoles of P/mg 10 min) for the corresponding fraction. This may be due to the more complete disruption of nuclei by sonication and, consequently, to the extraction of more protein.

For further purification, Teraoka et al. (1975) used phosphocellulose chromatography and gel filtration, while Levin and Zimmermann (1976) applied chromatography on phosphocellulose and on Sulfopropyl Sephadex. In our procedure the enzyme is further purified by affinity chromatography on DNA agarose, a method described by Schaller et al. (1972) for the isolation and fractionation of the DNA specific enzymes of *E. coli*. This procedure also proved to be highly efficient in this case, as it allowed the elimination of the majority of the contaminating proteins in one step. The pooled fractions containing poly-

nucleotide kinase activity represented 1–5% of the amount of protein and 50–70% of the total activity loaded on the column. The average value of purification in this step was 70-fold as compared to the crude extract.

The specific activity of the purified enzyme was 10–13 nmoles of P/mg/10 min. This value falls in the same order of magnitude as the specific activities of the phosphocellulose fractions reported by Teraoka et al. (1975) (30.8 nmoles of P/mg/10 min) and by Levin and Zimmermann (1976) (33 nmoles of P/mg/10 min).

The purification data of a representative experiment are shown in Table 1.

Table 1

Purification of polynucleotide kinase from rat liver nuclei
In this experiment 278 g of rat liver were used

Fraction	Protein mg	Specific act. nmoles of P mg/10 min	Purification	Yield	
				% protein	% activity
Crude extract	45.4	0.39	(1)	(100)	(100)
Pooled DNA agarose fractions	1.0	12.38	32×	2.2	70

The time dependence of the activity of the purified enzyme was linear up to 30 min, which suggests that the enzyme did not contain significant amounts of nuclease contamination. Under the conditions of the polynucleotide kinase assay, the purified enzyme preparation did not express any ATPase activity, i.e. no $^{32}P_i$ was formed from [γ-^{32}P]-ATP (result not shown).

The stability of the enzyme purified on DNA agarose was very poor: when stored at 4 °C, the activity was lost within one week. According to our experience this was mainly due to the low protein concentration of this fraction. The stability of the enzyme could be increased by concentrating the solution and/or by the addition of 5% (v/v) glycerol or of 1–10 μM ATP to the preparation.

Optimal conditions of the polynucleotide kinase assay

The pH optimum of both the crude extract and the purified enzyme was found to be pH 5.5, in agreement with Ichimura and Tsukada (1971), Teraoka et al. (1975) and Levin and Zimmermann (1976) (not shown).

Dependence upon ionic strength. As the activity of both T4-induced and rat liver polynucleotide kinases has been shown to depend markedly upon ionic strength (see Lillehaug, Kleppe, 1975b and Levin, Zimmermann, 1976, respectively), the dependence of the activity on NaCl concentration was studied in detail. In these experiments the buffer concentration of the reaction mixture was decreased to 10 mM. The enzyme solution was freed from eluent NaCl by diafiltration in an Amicon Model 8MC cell (PM-10 membrane) so that the addition of the enzyme increased the NaCl concentration of the reaction mixture by at most 0.015 M.

The results are shown in Fig. 1. Increasing the concentration of NaCl from 0 to 0.15 M resulted in a ca. 20% activation. Maximal kinase activity was measured at 0.10–0.15 M NaCl; higher salt concentrations inhibited the activity.

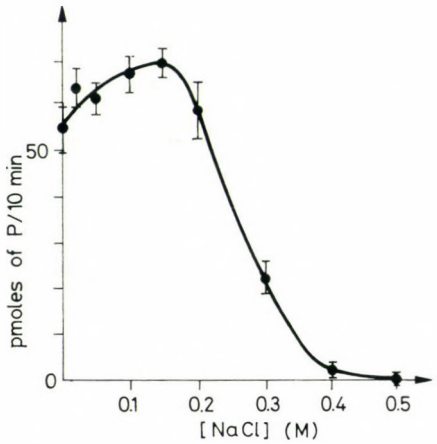

Fig. 1. Dependence of polynucleotide kinase activity on NaCl concentration. Besides NaCl, the reaction mixtures contained 10 mM acetate buffer (pH 5.5), 100 μM [γ-^{32}P]-ATP, 1 mM 5'-OH-DNA nucleotide and 30 μg purified polynucleotide kinase that had been freed from eluent NaCl by diafiltration. (For other components see Materials and methods.) The reaction mixtures were incubated for 10 min at 37 °C. Each point is the mean of 3 parallels, with $\pm$S.D. indicated

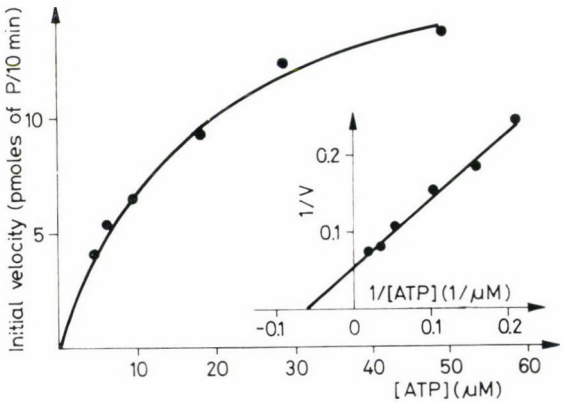

Fig. 2. Determination of the apparent K_M of polynucleotide kinase for ATP at a fixed concentration of 5'-OH-DNA of 1 mM. Incorporation for 10 min at 37 °C was measured. The reaction mixtures contained 3.24 μg of purified enzyme, 1 mM 5'-OH-DNA, 4.73–49.13 μM [γ-^{32}P]-ATP (exact ATP concentrations were determined spectrophotometrically), 0.1 M NaCl, 10% glycerol and other components as specified in Materials and methods. Each point is the mean of 3 parallels. The inset shows the double reciprocal plot of the same data. The best fitting straight line was determined by linear regression analysis

A similar type of dependence on ionic strength has been reported for the rat liver enzyme by Levin and Zimmermann (1976), but the maximal activity was measured at 0.05 M NaCl. The cause of this discrepancy is not known; one possible explanation is that a different rat strain, PVG/c was used, while Levin and Zimmermann worked on Sprague–Dawley rats which may contain a slightly different enzyme.

Determination of the apparent K_M values for ATP and 5'-OH-DNA. The dependence of polynucleotide kinase activity on ATP concentration was studied at a fixed concentration of 5'-OH-DNA of 1 mM and dependence on 5'-OH-DNA concentration was measured at a fixed concentration of ATP of 100 μM, in the presence of 0.1 M NaCl. Before each experiment, the conditions of the measurement of the initial velocity were established by time dependence measurements. The apparent K_M for ATP at 1 mM 5'-OH-DNA was found to be 15.8 μM (Fig. 2) and that for 5'-OH-DNA at 100 μM ATP to be 46.2 μM (Fig. 3).

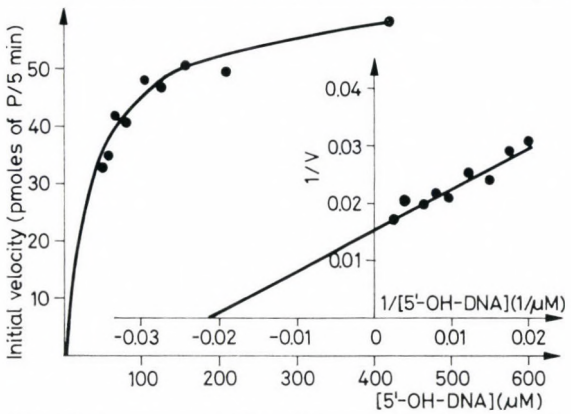

Fig. 3. Determination of the apparent K_M of polynucleotide kinase for 5'-OH-DNA at a fixed concentration of ATP of 100 μM. Incorporation for 5 min at 37 °C was measured. The reaction mixtures contained 13.6 μg of purified enzyme, 100 μM [γ-^{32}P]-ATP, 51–418 μM 5'-OH-DNA (exact DNA concentrations were determined spectrophotometrically), 0.1 M NaCl and other components as specified in Materials and methods. Each point is the mean of 3 parallels. The inset shows the double reciprocal plot of the same data. The best fitting straight line was determined by linear regression analysis

So far, apparent K_M values for rat liver polynucleotide kinase have been reported by Teraoka et al. (1975): 2.2 μM for ATP at 289 μM DNA and 35.5 μM for DNA at 8.3 μM ATP, measured in the absence of salts. Unfortunately these results are not directly comparable with ours as in the above report DNA concentrations are expressed in terms of DNA nucleotide rather than 5'-OH-termini. Recently Pheiffer and Zimmermann (1979) reported K_M values of 6 μM for ATP and 24 μg/ml (= 73 μM) for DNA, measured in the presence of 0.1 M KCl.

On the basis of these measurements, 100 μM ATP and 1 mM 5'-OH-DNA were chosen as saturating substrate concentrations for the standard polynucleotide kinase assay.

Heat inactivation of polynucleotide kinase

Heat inactivation experiments were carried out for two reasons: first, to get an insight into the reaction mechanism of a mammalian polynucleotide kinase by studying the protective effect of the individual substrates against heat inactivation; second, to decide whether protection by DNA, established in the first type of experiments, is due only to specific enzyme–substrate interactions or other, less specific interactions also take place. In order to answer the latter question, heat inactivation in the presence of either 5'-OH- or 5'-P-DNA was studied.

The setup of the heat inactivation experiments was the following. The enzyme was preincubated at 37 °C in an incomplete reaction mixture containing only the substrate to be studied. After different preincubation times the residual activity was determined by completing the reaction mixture with the other substrate and allowing phosphorylation to proceed for 10 minutes. (In each experiment a control series was also prepared in which the enzyme was preincubated for different times without substrates, then the residual activity was measured by adding both substrates and allowing phosphorylation to proceed for 10 minutes.) The reaction was stopped and acid-insoluble radioactivity was determined as described in Methods. Residual activities are expressed as the percentage of the enzyme activity measured after preincubation at 37 °C for 2 minutes (i.e. the usual preincubation time allowing the components to warm up).

Heat inactivation experiments for the determination of the reaction mechanism. T4-induced polynucleotide kinase has been reported to react according to an ordered or random reaction mechanism depending upon the absence or presence, respectively, of salts or polyamines in the medium (Lillehaug, Kleppe, 1975a, b). It seemed of interest to study whether this unusual change occurs in the case of the rat liver enzyme as well. We therefore carried out heat inactivation experiments, preincubating the enzyme at 37 °C in the presence of either ATP or 5'-OH-DNA at a very low (0.06 M) and a moderately high (0.2 M) NaCl concentration, reasoning that if the reaction mechanism is of the random type, either substrate may be expected to protect the enzyme, while if it is ordered, only the first substrate will give protection. After preincubation for different times with one substrate, phosphorylation was started by the addition of the other substrate (in the control series both substrates were added) and residual activity was determined.

Figure 4 demonstrates that at both 0.06 M (Fig. 4A) and 0.2 M (Fig. 4B) NaCl, ATP as well as 5'-OH-DNA were able to protect polynucleotide kinase against heat inactivation. (The difference between the rates of inactivation of the control samples in Figs 4A and 4B may be due to the difference between the enzyme concentrations in the preincubation mixtures (65 μg/ml in Fig. 4A and only 16 μg/ml in Fig. 4B), as the enzyme was found to be very labile in dilute solutions.)

Protection by 5'-OH-DNA was studied also at 0.3 M NaCl, a partially inhibitory NaCl concentration. In this case, no protection was observed (result not shown).

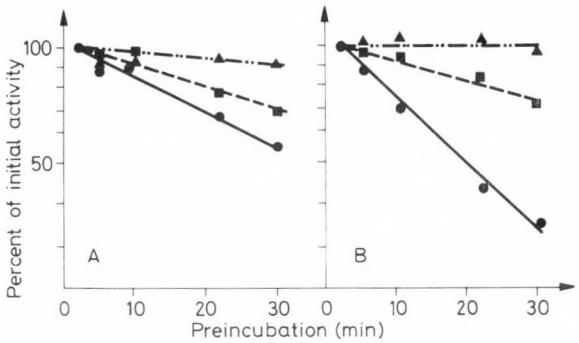

Fig. 4. Heat inactivation of polynucleotide kinase at two different sodium chloride concentrations, in the presence of either [γ-^{32}P]-ATP or 5'-OH-DNA or in the absence of substrates at 37 °C. Purified polynucleotide kinase freed from NaCl by diafiltration was preincubated at 37 °C under the following conditions: (A) 0.06 M NaCl, 65 μg/ml enzyme and either 120 μM [γ-^{32}P]-ATP (▲-·-·-▲) or 1.5 mM 5'-OH-DNA (■- - -■) or no substrate (●——●); (B) 0.2 M NaCl, 16 μg/ml enzyme and either 125 μM [γ-^{32}P]-ATP (▲-·-·-▲) or 1 mM 5'-OH-DNA (■- - -■) or no substrate (●——●). After the preincubation times indicated, the preincubation mixtures were completed to give the following final concentrations: (A) 0.04 M NaCl, 100 μM [γ-^{32}P]-ATP, 1 mM 5'-OH-DNA; (B) 0.15 M NaCl, 100 μM [γ-^{32}P]-ATP, 0.8 mM 5'-OH-DNA (for other complements see Materials and methods) and phosphorylation was allowed to proceed for 10 min. Each point is the mean of 3 parallels

These results suggest that under these conditions rat liver polynucleotide kinase reacts according to a random reaction mechanism which is not affected by variations in NaCl concentrations. Recently, Pheiffer and Zimmermann (1979) published a detailed kinetic study according to which the enzyme follows a random sequential mechanism.

Heat inactivation experiments for the characterization of the bonds between polynucleotide kinase and DNA. It was an open question whether protection against heat inactivation by DNA was due only to specific enzyme–substrate interactions or whether other parts of DNA also participated in it by way of less specific interactions with the enzyme. Therefore it seemed promising to investigate whether 5'-P-DNA, which can be supposed to interact with the enzyme only non-specifically, is able to protect the enzyme against heat inactivation.

For these experiments, a DNA substrate preparation was divided into two parts after digestion with DNase I. The dephosphorylation step was then omitted for one of these, otherwise the two parts were treated identically. In this way 5'-OH- and 5'-P-DNA preparations of identical molecular characteristics were obtained. When tested separately, the incorporation of ^{32}P into the 5'-P-DNA preparation was one tenth of that measured in the 5'-OH-DNA preparation, which is accounted for by the 5'-OH-termini originally present in the high molecular weight DNA used.

The experiment was carried out at optimal (0.15 M) NaCl concentration. Polynucleotide kinase was preincubated at 37 °C with either 5'OH-DNA (specific and non-specific interactions) or with 5'-P-DNA (only non-specific interactions)

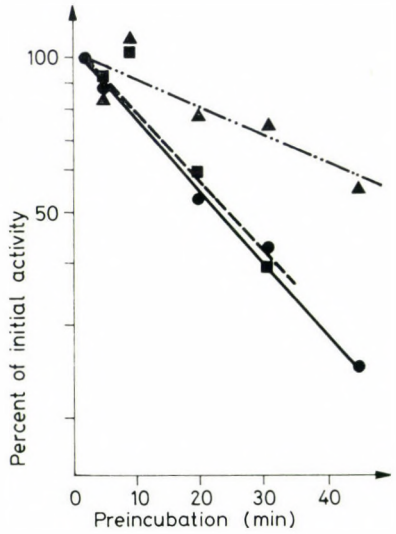

Fig. 5. Heat inactivation of polynucleotide kinase at 0.15 M NaCl, in the presence of either 5'-OH-DNA or 5'-P-DNA or without DNA at 37 °C. Purified polynucleotide kinase freed from NaCl by diafiltration was preincubated at 37 °C under the following conditions: 0.15 M NaCl, 62 μg/ml enzyme and either 1.5 mM 5'-OH-DNA (▲–···–▲) or 1.5 mM 5'-P-DNA (■– – –■) or no DNA (●——●). After the preincubation times indicated, the preincubation mixtures were completed to give the following final concentrations: 0.15 M NaCl, 200 μM [γ-^{32}P]-ATP, 1 mM 5'-OH-DNA, 1 mM 5'-P-DNA (for other components see Materials and methods), and phosphorylation was allowed to proceed for 10 min. Each point is the mean of 3 parallels

After different preincubation times, the preincubation mixtures were completed with the other DNA species plus [γ-^{32}P]-ATP (in the control series, with both DNA species plus [γ-^{32}P]-ATP) and residual activity was measured. In this way, residual activities were measured under identical conditions in each of the three series, i.e. in the presence of both 5'-OH- and 5'-P-DNA.

The result of this experiment does not substantiate the assumption of non-specific interactions between polynucleotide kinase and DNA. As it is seen in Figure 5, at 0.15 M NaCl 5'-P-DNA does not, while 5'-OH-DNA does protect the enzyme.

This result indicates that under the conditions applied 5'-P-DNA is not bound by the enzyme, or if it is, the dissociation constant of the resulting complex is too high for protection to occur. 5'-OH-DNA was found to protect the enzyme at 0.06, 0.15 and 0.2 M NaCl (Figs 4A, 5 and 4B, respectively), but not at 0.3 M NaCl (not shown). Thus, protection against heat inactivation is due solely to specific enzyme–substrate interactions and is possible only at NaCl concentrations which permit the enzyme to bind 5'-OH-DNA. Our results, of course, do not preclude the possibility of some binding of 5'-P-DNA to the enzyme; however, this interaction must be too weak to be detected by heat inactivation experiments.

We consider that the results described here all reflect the same underlying fact, i.e. that between 0.2 and 0.3 M NaCl concentrations the ability of the enzyme to form a complex with 5'-OH-DNA is lost. It is between these NaCl concentrations that the enzyme is eluted from DNA agarose, in which DNA containing many 5'-OH-termini is immobilized; that enzyme activity steeply decreases (Fig. 1) and protection against heat inactivation ceases (Fig. 4B and result not shown, respectively).

The authors are grateful to Miss Zsuzsanna Monostori for her valuable technical assistance.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (4), pp. 287–293 (1980)

A New Method for the Isolation of Aminoacylase from Mammalian Kidneys

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(Received December 23, 1979)

Aminoacylase (E.C. 3.5.1.14) was isolated from the kidneys of different mammalian species (horse, cattle, rabbit and pig) by extracting the organ with water and subjecting the extract to heat treatment at 70 °C for 10 min, then, after having removed denatured proteins by fractionating those remaining in the solution by ammonium sulfate. The enzyme obtained in this way can either be used directly for practical purposes (e.g. preparation of immobilized aminoacylase) or further purified by chromatography. For the further purification of porcine kidney aminoacylase we applied a combination of ion exchange chromatography and gel filtration.

Introduction

Mammalian aminoacylase (E.C. 3.5.1.14) is usually isolated from porcine kidney and for this reason it is the catalytic properties and structure of only this enzyme that are known. Aminoacylase has not been isolated from the kidneys of other mammalian species.

The first data in the literature about procine kidney aminoacylase were published by Schmiedeberg (1881); however, detailed investigations started only at the end of the forties of this century. The first preparation, having a specific activity about 4 times higher than that of the extract of the organ, was prepared by Fodor et al. (1949) by ethanol fractionation of the aqueous extract of porcine kidney. A few years later Birnbaum et al. (1952) elaborated a method for the isolation of the enzyme, based on pH, ammonium sulfate and acetone fractionations, which resulted in a 30-fold purification approximately. Essentially the same method was further developed by Bruns and Schulze (1962) who, by applying an additional ion-exchange chromatographic step on DEAE-cellulose, reached a 300-fold purification approximately and obtained a product that proved to be electrophoretically homogeneous. The method of Tschen-U Tsi and Orechowitsch (1957) is also worth mentioning: these authors started their preparation from the acetone powder of porcine kidney and by an appropriate combination of ammonium sulfate and acetone fractionations and heat treatment could reach a 70–100-fold purification.

In the course of investigations of the industrial applicability of porcine kidney aminoacylase, we aimed at the elaboration of a method by which an enzyme preparation of high specific activity could be prepared at an industrial scale. In this

work our previous experience showing that the enzyme is relatively heat-stable in the water extract of porcine kidney offered a sound basis. In accordance with this experience, the first step of our procedure is the heat treatment of the water extract of porcine kidney. We also tested the possibility of applying our method to the isolation of aminoacylase from the kidneys of other mammalian species.

Materials

Porcine kidney was freshly collected in a slaughterhouse and stored frozen at −20 °C until use.

Ammonium sulfate, p.a. quality was a commercial preparation (Reanal).

DEAE-Sephadex A-50 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

All the other chemicals used were reagent grade commercial products (Reanal).

Methods

Protein concentration was determined according to Lowry et al. (1951) as modified by Schacterle and Pollack (1973). Bovine serum albumin (Serva Fein Biochemica, Heidelberg, FRG) was used as standard for the calibration curve.

Aminoacylase activity was measured by determining the amount of L-methionine liberated in the reaction as described previously (Szajáni et al., 1980).

Polyacrylamide gel electrophoresis was performed in the discontinuous buffer system described by Ornstein (1964) and Davis (1964) in a Model 69 type equipment manufactured by Reanal. The total monomer concentration (acrylamide + + N,N'-methylenebisacrylamide) in the gels was 10%, the concentration of N,N'-methylenebisacrylamide being 5% of the total monomer concentration. To detect eventual contaminations, we overloaded the gels by electrophoresing 400 μg instead of the usual 50–100 μg of protein per gel. The samples were applied onto the gels in 20% solution of sucrose. Electrophoretic runs were made at a constant current of 5 mA/gel for 2 hours. The gels were stained in 1% solution of amidoblack 10B dissolved in 7% acetic acid.

Results

Heat treatment of the aqueous extract of porcine kidney

To determine the optimal temperature and time of heat treatment, 2-liter volumes of the aqueous extract of porcine kidney were kept at 60–80 °C for 5–15 minutes. After the heat treatment the cooled suspensions were centrifuged, and the specific activity of aminoacylase was determined in the supernatant. Our

Table 1

Heat treatment of the aqueous extract of porcine kidney
In the experiment 2-liter volumes of extract were heat-treated

Temperature (°C)	Time (min)	*Purification	Yield (%)
60	10	1.5	46
60	15	3.8	55
70	10	2.8	28
75	5	2.6	23
80	5	1.3	9

* Purification = specific activity after heat treatment/specific activity in the extract

results are summarized in Table 1. Although both maximal purification and minimal loss can be achieved by heat treatment at 60 °C for 15 min, the high lipid content of the supernatant presents a disadvantage in the further purification steps. For this reason we chose to carry out heat treatment at 70 °C for 10 minutes.

A new method for the isolation of porcine kidney aminoacylase

Extraction. 15 kg of porcine kidney, either fresh or frozen and slowly brought to room temperature was trimmed free of fat and connective tissue and homogenized in several portions in 2 volumes (v/w) of cold (2–4 °C) distilled water using a blendor in several portions. The homogenate was stirred mechanically for 1 hour in an ice bath, centrifuged for 30 min at $2800 \times g$ and the pellet was discarded.

Heat treatment. The supernatant was heated to 70 °C in a water bath and kept at this temperature for 10 min, then cooled to 15–18 °C in an ice bath. The solution was then centrifuged at the same temperature at $2800 \times g$ for 30 min. If the supernatant remained slightly turbid, it was clarified by filtration through paper.

Precipitation by ammonium sulfate. To the filtrate 266 g of ammonium sulfate per liter was added (Birnbaum et al., 1952). The suspension was left to stand at +4 °C for 16 hours. The supernatant was then decanted and the remaining thick suspension was centrifuged at $2800 \times g$. The supernatant was discarded. The pellet was dissolved in a small amount of cold (0–4 °C) water and freed from sulfate by dialysis against distilled water. After dialysis the solution was filtered through a G-4 glass filter and lyophilized.

Purification by ion exchange. To 50 g of DEAE-Sephadex A-50 equilibrated with 0.05 M K-phosphate buffer (pH 7.0), 3 g of aminoacylase, purified according to the above procedure and dissolved in 1 liter of equilibrating buffer, was added. The mixture of the gel and the enzyme solution was stirred for 3 hours in an

ice bath and left to stand for 16 hours at +4 °C. Next day the gel was washed with 3×1 liter of buffer. The individual washes were carried out by stirring the mixture of the gel and the buffer for 1 hour in an ice bath, followed by filtration on a G-3 glass filter. Finally the gel carrying the bound protein was suspended in 1 liter of fresh buffer and packed into a column (5.3×55 cm). The material was eluted by 0.05 M K-phosphate buffer (pH 7.0) containing 0.3 M NaCl. The optimal NaCl concentration had been determined in earlier gradient experiments. The flow-rate was 200 ml/hour and fractions of 100 ml were tested for enzyme activity and protein content. The active fractions were pooled and protein was precipitated by the addition of 266 g of ammonium sulfate per liter. Until further use, the suspension was stored at +4 °C.

Gel filtration. 25 ml of aminoacylase which had been purified by ion exchange and precipitated by ammonium sulfate (2.28 mg/ml) was centrifuged and the pellet was dissolved in 9 ml of 0.05 M K-phosphate buffer (pH 7.0). The solution was loaded on a 2.4×43 cm Sephadex G-200 column which had been washed by the above buffer. The same buffer was used for elution. The flow rate was 60 ml/hour and 5 ml fractions were collected. The fractions were tested for enzyme activity, protein content and conductivity (to check the absence of ammonium sulfate). The active fractions were pooled and ammonium sulfate (266 g/liter) was added. The suspension was stored at +4 °C.

The data of a typical experiment are summarized in Table 2. As seen from the Table, a nearly 80-fold purification was achieved by our method relative to

Table 2

Isolation of aminoacylase from porcine kidney
In the experiment 15 kg kidney was processed

Purification step	Volume (ml)	Total activity (units × 10^{-7})	Yield (%)	Total protein (g)	*Specific activity (units/mg)	Purification
Aqueous extract (2800×*g* supernatant)	24000	11.6	100	916.8	127	1.0
Heat treatment (70 °C, 10 min, 2800×*g* supernatant)	9600	3.2	27.6	96	340	2.67
$(NH_4)_2SO_4$ fractionation (266 g/l); dissolved precipitate	500	2.1	18.1	7.4	2840	22.36
Lyophilization	—	1.97	16.7	7.2	2735	21.5
Ion exchange (DEAE-Sephadex A-50)	720	0.86	7.4	1.64	5240	41.26
Gel filtration (Sephadex G-200)	960	0.73	6.3	0.73	10000	78.74

* Measured at 25 °C

the specific activity measured in the original extract. The specific activity of our best preparations was 11 000 ± 1 000 units/mg protein, measured at 25 °C. This value represents about 80–85% of the activity measurable at 37 °C. The gel electrophoretic picture of our preparation is shown in Figure 1.

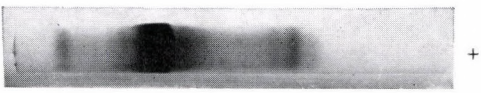

Fig. 1. Polyacrylamide gel electrophoresis of purified porcine kidney aminoacylase. 400 μg of protein was applied to the gel. For the details of the procedure see the text

The specific activities of porcine kidney aminoacylase preparations purified by different method are compared in Table 3.

Table 3

Comparison of the specific activities at 37 °C of porcine kidney aminoacylase preparations purified by different methods

Reference	[1]Specific activity (units/mg protein)
Birnbaum et al. (1952)	[2]4640
Tschen-U Tsi and Orechowitsch (1957)	[2]about 15000
Bruns and Schulze (1962)	[3]15600
Present report	13300

[1] Values calculated for N-acetyl-L-methionine as substrate. One unit is that amount of enzyme which catalyzes the liberation of 1 μmole of L-methionine in 1 hour

[2] Specific activity originally expressed as units/mg N was converted by using a factor of 1 mg of protein = 0.16 mg of N (Birnbaum, 1955)

[3] Using N-acetyl-DL-methionine as substrate

Table 4

Isolation of aminoacylase from the kidneys of different mammals

The data in the Table refer to the state before purification by chromatography

Species	Specific activity (units/mg protein)		Purification
	Extract	After purification	
Horse	8	319	39.9
Cattle	7	791	113
Rabbit	26	946	36.4
Pig	127	2735	21.5

Isolation of horse, bovine and rabbit kidney aminoacylases

We studied the possibility whether our method for the isolation of porcine kidney aminoacylase is applicable for the isolation of kidney aminoacylases from other mammalian species. We used horse, bovine and rabbit kidneys; the chromatographic steps were omitted. Our results are summarized in Table 4. It is evident that the method is applicable also in the case of the above mammals.

Discussion

The methods earlier described for the isolation of porcine kidney aminoacylase employed fractionation by organic solvents (acetone, ethanol) (Fodor et al., 1949; Birnbaum et al., 1952; Tschen-U Tsi, Orechowitsch, 1957; Bruns, Schulze, 1962), or used the actone powder of the kidneys as starting material (Tschen-U Tsi, Orechowitsch, 1957). With such treatments denaturation must be prevented by intensive cooling, etc., and this sets a limit to increasing the amount of starting material. It may not be by chance that Birnbaum et al. (1952) started from 2.5 kg, Tschen-U Tsi and Orechowitsch (1957) from 2 kg and Bruns and Schulze (1962) from 0.75 kg of kidney. From a practical point of view, the inflammability and expensiveness of organic solvents are also disadvantageous. It was with the aim of avoiding these disadvantages that we worked out our new method which is based on the relative heat stability of aminoacylase in the aqueous extract of porcine kidney. This method makes it possible to use high amounts of starting material: we processed 15–30 kg of porcine kidney at a time and obtained a product whose specific activity was close to that of the best preparations described in the literature (cf. the data in Table 3), and contained a minimal amount of contaminating protein (Fig. 1). Also, there is no theoretical limit to further increase the amount of kidney processed at a time.

Another advantage of our method is that it can be used for the isolation of aminoacylase from the kidneys of other mammalian species, as exemplified by equine, bovine and rabbit aminoacylases. An investigation of the catalytic properties of the kidney aminoacylases of these species is in progress.

The author expresses his gratitude to Dr Gertrud Szabolcsi for her valuable comments given in the course of the preparation of the manuscript and to Miss Irén Huber for her conscientious technical assistance.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (4), pp. 295–303 (1980)

Preparation and Practical Utilization of a Highly Active Immobilized Form of Porcine Kidney Aminoacylase

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(Received October 15, 1979 and in revised form January 18, 1980)

Experiments were carried out with the aim of preparing an immobilized form of porcine kidney aminoacylase (E.C. 3.5.1.14). The immobilization of the enzyme via covalent bonds was most successfully achieved on solid supports with carboxyl functional groups activated by water-soluble carbodiimides.

Introduction

The optical isomers of amino acids may be separated from their synthetically prepared racemates starting from the N-acetyl derivatives of the racemates by the application of acylase enzymes, the characteristic feature of which is a high stereospecificity. Because of their great practical significance, the preparation and industrial–technological applications of immobilized aminoacylases were subject to intensive research already in the sixties of this century, first of all in Japan (Kirimura, Yoshida, 1966; Kyowa Hakko Kogyo Kabushiki Kaisha, 1966; Tosa et al., 1966a, b, 1967a, b, 1969a, b). These intensive studies resulted in the elaboration of the first industrial process, based on immobilized enzyme, for the separation of optical isomers from racemic amino acids, and the experimental plant based on this procedure was built (Chibata et al., 1972).

In the research cited above of mostly industrial orientation, aminoacylases of microbial origin (mainly *Aspergillus oryzae*) were applied. Only few data have been available about the immobilization of porcine kidney aminoacylase. Its first immobilized derivative was prepared by Mitz and Schlueter in 1959, who bound the enzyme to DEAE-cellulose via ionic bonds. Porcine kidney aminoacylase immobilized via covalent bonds was obtained by Ohno and Stahmann (1971) and Masková et al. (1973).

In the course of our studies on the industrial applicability of porcine kidney aminoacylase, we immobilized the enzyme on solid support of different types via

Abbreviations: CM-, carboxymethyl; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate; EDAPC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodimide; PAB-, *p*-aminobenzyl.

covalent chemical bonds between the functional groups of the solid support and those of the enzyme. In these studies it was established that the enzyme can be immobilized most successfully on solid supports with carboxyl functional groups activated by water-soluble carbodiimides.

In the present study we give an account on our immobilization experiments and on the practical utilization of the immobilized aminoacylase.

Materials

Aminoacylase, isolated from porcine kidney, salt-free lyophilized commercial preparation (Reanal Factory of Laboratory Chemicals, Budapest, Hungary). Specific activity: 2500 ± 200 units/mg protein.

Solid supports

Akrilex AH, bead polymer of polyacrylamide matrix with acid hydrazide functional groups, produced by Reanal, the grain size and binding capacity ($-NH-NH_2$ content) of which were the following:

Akrilex	AH- 4	50 – 100 μ	6.0 ± 0.2 meq/g
	AH- 30	50 – 100 μ	4.4 ± 0.3 meq/g
	AH-100	100 – 320 μ	4.9 ± 0.2 meq/g

Akrilex C, bead polymer of polyacrylamide matrix with carboxyl functional groups, manufactured by Reanal, the grain size and binding capacity (−COOH content) of which were the following:

Akrilex	C- 4	50 – 100 μ	4.8 ± 0.2 meq/g
	C- 30	50 – 100 μ	5.7 ± 0.3 meq/g
	C-100	100 – 320 μ	6.2 ± 0.3 meq/g

Bio-Gel CM-100, product of BIO-RAD Laboratories (Richmond, Calif.). A gel with a grain size of 100 – 200 mesh (wet), with a binding capacity of 6.0 ± 0.3 meq/g dry weight was used as solid support.

Enzacryl AA and -AH, products of Koch-Light Laboratories Ltd. (Colnbrook, Bucks, England).

Polymaleic anhydride, purchased from MERCK (Darmstadt, FRG). Cross-links were formed by butanediol-(1,4)-divinylether.

PAB-cellulose, Serva Feinbiochemica (Heidelberg, FRG) and Reanal preparations were used.

1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide were from Serva and Sigma Chemical Company (St. Louis, USA).

N-acyl-amino acids and all the other chemicals used were the reagent grade commercial products of Reanal.

Methods

Determination of protein concentration was carried out according to Lowry et al. (1951), as modified by Schacterle and Pollack (1973). The amount of immobilized protein was determined indirectly as the difference between the amount of protein originally added to the reaction mixture and that remaining in the supernatant after the immobilization procedure and in the washes.

Measurement of aminoacylase activity: the activity of both soluble and immobilized enzyme preparations was determined by the ninhydrin reaction of L-methionine liberated in the course of the deacetylation of N-acetyl-L-methionine (Moore, Stein, 1948; Spies, 1957) as described earlier (Szajáni et al., 1980; Szajáni, Fölser, 1976).

Immobilization procedures: porcine kidney aminoacylase was bound via covalent chemical bonds to solid supports of different types. The following classification of immobilization processes is essentially based on the system of Barker and Kennedy (1975).

1. Formation of covalent bonds via diazonium groups derived from aromatic amino groups

1.1. Immobilization on PAB-cellulose: the solid support was diazotized according to the instructions of Serva Entwicklungslabor (1962). The immobilization procedure was the following:

2 g diazotized PAB-cellulose and 200 mg aminoacylase were stirred in 20 ml 0.05 M sodium phosphate buffer (pH 7.0) at 0 °C for 8 hours, then allowed to stand in the refrigerator (+4 °C) for 16 hours. The suspension was then filtered and the unreacted diazonium groups were blocked by β-naphtol.

1.2. Immobilization on Enzacryl AA: Enzacryl AA was diazotized according to the instructions of the manufacturer (Koch-Light Ltd., 1970). In the course of immobilization, the experiences of Masková et al. (1973) about the immobilization of porcine kidney aminoacylase were also taken into consideration.

100 mg diazotized Enzacryl AA and 2.5 mg aminoacylase were stirred in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5) at 0 °C for 2×8 hours, alternated with allowing to stand in the refrigerator (+4 °C) for 2×16 hours.

1.3. Immobilization on cellulose-Bismarck brown adsorptive complex: in these experiments we followed the procedure described by Gray et al. (1974) for the immobilization of different enzymes.

500 mg m-diaminobenzene and 1 g cellulose powder were suspended in 20 ml 1 M hydrochloric acid and stirred with 20 ml 2% solution of sodium nitrite at 0 °C for 30 minutes. The solid support was washed with 3×50 ml 0.2 M acetate buffer (pH 5.0). 10 mg aminoacylase dissolved in 50 ml 0.2 M acetate buffer (pH 5.0) were then added and the mixture was stirred for 2 hours at 0 °C. Finally 20 ml β-naphtol solution saturated at 0 °C was added, followed by stirring for 30 minutes at 0 °C.

2. Formation of covalent bonds via isothiocyanate groups raised by thiophosgene treatment of amino groups

2.1. Immobilization on Enzacryl AA: the activation of Enzacryl AA by thiophosgene was carried out according to the instructions of the manufacturer (Koch-Light Ltd., 1970).

500 mg Enzacryl AA activated by thiophosgene and 12.5 mg aminoacylase were stirred in 2.5 ml 0.1 M borate buffer (pH 8.5) at 0 °C for 2×8 hours alternated with keeping in the refrigerator (+4 °C) for 2×16 hours.

3. Formation of covalent bonds via azide groups raised by treatment of hydrazides with nitrous acid

3.1. Immobilization on Enzacryl AA: the solid support was activated according to the instructions of the manufacturer (Koch-Light Ltd., 1970), in acidic medium by sodium nitrite.

500 mg activated support and 50 mg aminoacylase were stirred in 25 ml 0.05 M sodium phosphate buffer (pH 7.0) at 0 °C for 2×8 hours alternated with allowing to stand in the refrigerator (+4 °C) for 2×16 hours.

3.2. Immobilization on solid supports of Akrilex AH type: for the immobilization of porcine kidney aminoacylase on solid supports of Akrilex AH type, an adaptation of the method for the preparation of CM-cellulose-protein conjugates, described by Micheel and Evers (1949), was used.

Solid supports of the Akrilex AH type were activated in acid medium by sodium nitrite. After activation, 500 mg solid support and 100 mg aminoacylase were stirred in 25 ml of 0.1 M potassium phosphate buffer (pH 7.0) in a refrigerator (+4 °C) for 24 hours.

4. Formation of covalent bonds via carboxyl groups activated by water-soluble carbodiimides

4.1. Immobilization on Bio-Gel CM-100: The solid support was activated according to Mosbach's method (Mosbach, 1970; Mosbach, Mattiasson, 1970).

1 g xerogel was suspended in 50 ml 0.1 M potassium phosphate buffer (pH 7.0) and stirred for 20 min at 0 °C. 500 mg CMC and, after 1 minute of stirring, 1 g aminoacylase dissolved in 20 ml buffer were added and the suspension was stirred for 24 hours in the refrigerator (+4 °C).

4.2. Immobilization on solid supports of Akrilex C type: the carboxyl groups of these solid supports were activated by means of water-soluble carbodiimides, a method based on that of Mosbach (Mosbach, 1970; Mosbach, Mattiasson, 1970). For activation in neutral medium, CMC or EDAPC was used in amounts stoichiometric with that of carboxyl groups.

To 1 g xerogel suspended in 50 ml 0.1 M potassium phosphate buffer (pH 7.0), 2 g CMC or 1 g EDAPC dissolved in 25 ml cold (+4 °C) buffer was added at 0 °C, with constant stirring. After 10 minutes of stirring, 500 mg

aminoacylase dissolved in 25 ml cold (+4 °C) buffer was added. The total reaction time at 0–4 °C was 48 hours, from which the reaction mixture was stirred for 2×6 hours.

5. Formation of covalent bonds with polymers containing anhydride groups

5.1. Immobilization on polymaleic anhydride: the general procedure recommended by MERCK was followed.

1 g solid support was suspended in 25 ml 0.05 M potassium phosphate buffer (pH 7.0) at +4 °C. To the suspension 500 mg aminoacylase dissolved in 5 ml cold (+4 °C) buffer was added. The pH of the reaction mixture was kept between 8 and 9 by the continuous addition of 1 N NaOH. After alkali consumption stopped (ca. 3 hours), the suspension was filtered.

After the termination of the coupling reactions the immobilized enzymes were filtered or centrifuged and washed first by the buffer used during immobilization, then by buffer containing 0.5–1.0 M NaCl and then again by buffer, in order to remove the noncovalently bound protein fraction. Immobilized enzymes were stored in buffered suspension at +4 °C. Akrilex C-100-aminoacylase was lyophilized and stored in this form. Prior to lyophilization, buffer ions were removed by washing in water.

Quantitative determination of amino acids in water solution: the ninhydrin test (Moore, Stein, 1948; Spies, 1957) was used.

Results

The preparation of immobilized porcine kidney aminoacylase

The results of our immobilization experiments are summarized in Table 1. As shown by the data in the Table, the majority of our experiments resulted in catalytically active immobilized enzymes. Immobilized aminoacylase possessing the highest activity relative to dry weight (protein + solid support) was obtained by the application of Akrilex C-100 solid support activated by water-soluble carbodiimides (CMC or EDAPC). The activity of this product is higher than that of any immobilized aminoacylase prepared earlier. The material balance of a typical immobilization experiment is shown in Table 2.

The activity of Akrilex C-100-aminoacylase relative to dry weight was 6–10% of the activity of the soluble enzyme and its specific activity calculated on the basis of the amount of immobilized protein and enzyme activity was ca. 25–30% of that of the soluble enzyme (average of several experiments).

Resolution of racemic amino acids by Akrilex C-100-aminoacylase

By the application of our Akrilex C-100-aminoacylase preparation, the optical isomers of DL-alanine, DL-methionine and DL-valine were successfully

Table 1

Experiments on the immobilization of porcine kidney aminoacylase
The details of the individual procedures are described in the text

Solid support	Activating agent	Functional group of polymer participating in bond	Catalytic activity of product (unit/mg dry weight)	Immobilized activity: yield expressed as % of starting activity
p-Aminobenzylcellulose	HNO_2	Diazonium	Inactive	0
Enzacryl AA	HNO_2	Diazonium	0.14	1.05
Cellulose-Bismarck brown adsorptive complex	HNO_2	Diazonium	Inactive	0
Enzacryl AA	Thio-phosgene	Isothio-cyanate	Inactive	0
Enzacryl AH	HNO_2	Azide	0.03	0.06
Akrilex AH- 4	HNO_2	Azide	0.76	0.77
AH- 30			0.38	0.34
AH-100			0.31	0.36
Bio-Gel CM-100	CMC	Carboxyl	8.7	1.17
Akrilex C- 4	CMC	Carboxyl	0.39	0.07
C- 30			25.6	3.03
C-100			158.0	21.0
C-100	EDAPC	Carboxyl	202.0	25.0
Maleic anhydride butanediol-(1,4)-divinylether copolymer	—	Anhydride	0.28	0.001

Table 2

Material balance of a typical immobilization experiment
Immobilization was carried out in 0.1 M potassium phosphate buffer (pH 7.0) at 0—4 °C; Akrilex C-100 was used as solid support with CMC as activating agent. The ratio of the weights of the solid support, CMC and enzyme used was 1 : 2 : 0.5

Immobilized protein (%)	Immobilized activity (%)	Activity recovered in solution (%)	Activity lost (%)
76.5	21.0	20.5	58.5

separated, starting from the N-acetyl derivatives of the racemates. The immobilized enzyme was filled into a column in order to permit continuous operation.

0.1 – 0.7 M solutions of N-acetyl-DL-amino acids (pH 8.5) were used as substrates. pH had been adjusted by the addition of NaOH solution. The substrate solution was passed through a 5.5 × 70 cm glass column containing 60 g

immobilized enzyme, at a flow rate of 1 liter/hour. The temperature in the column was 37 °C. The advancement of deacylation was monitored by the determination of the liberated L-amino acid by means of the ninhydrin reaction (Moore, Stein, 1948; Spies, 1957). In case if the deacylation of the L isomer was incomplete after one cycle, the solution was recirculated once more (after readjusting the pH).

After the complete deacylation of the L isomer, the pH of the solution containing the L amino acid plus the N-acetyl-D-amino acid was adjusted to 5.0 by the addition of glacial acetic acid, the solution was clarified by charcoal, filtered and the volume of the filtrate was reduced to ca. 1/6 of the original volume in vacuum at 40–60 °C. L-amino acid was then precipitated by ethanol added at a final concentration of 80%. Recrystallization was also done from 80% ethanol (Greenstein, 1957). Recovery amounted to min 70% of the theoretical yield.

Table 3

Characteristics of amino acids resolved by Akrilex C-100-aminoacylase

All the data in the Table refer to crystalline raw product

The conditions of the determination of the $[\alpha]_D^{20}$ values were the following (cf. Kolos, 1969):

alanine — 4% solution in 6 N HCl
methionine — 5% solution in 3 N HCl
valine — 4% solution in 6 N HCl

Nitrogen contents were determined by the micro-Kjeldahl method (cf. Kolos, 1969).

Amino acid	Optical isomer	$[\alpha]_D^{20}$		N content	
		Theoretical	Measured	Calculated	Found
Alanine	L	+14.6	+14.3	15.72	15.42
	D	−14.6	−14.5	15.72	15.48
Methionine	L	+23.2	+22.0	9.38	9.27
	D	−23.2	−22.0	9.38	9.25
Valine	L	+22.9	+23.7	11.95	11.82

Table 4

Amounts of L-amino acids obtained by Akrilex C-100-aminoacylase in 1 month

The experiments were carried out in a 5.5×70 cm column filled with 60 g of immobilized aminoacylase, which was operated continuously (day-and-night), at 37 °C. As substrate, the solution of the N-acetyl derivative of the racemate was used

Amino acid	Substrate concentration (M)	Amount obtained (kg/month)
Alanine	0.7	8.07
Methionine	0.7	11.66
Valine	0.1	3.97

N-acetyl-D-amino acid remaining in the original solution was purified by ion exchange chromatography on Varion KS ion exchanger (NIKE, Hungary).

The hydrolysis in hydrochloric acid of N-acetyl-D-amino acids and the isolation of D-amino acids from the hydrolysate were carried out according to Greenstein (1957). Minimally 50% of the theoretical yield was recovered.

Some characteristics of the products obtained by the above procedure are shown in Table 3.

In case of continuous day-and-night use, the enzyme loses 50% of its catalytic activity in ca. 2 months, i.e. this is the half-time of inactivation ($t_{1/2}$). The amounts of amino acids prepared in one column, i.e. by 60 g of immobilized enzyme in 1 month are shown in Table 4.

Discussion

Immobilization via covalent bonds was chosen for immobilizing porcine kidney aminoacylase as it is only in this case that a well-definable, stable molecular complex is formed which may be used in a wide range of substrate concentrations.

In the course of experimentation with solid supports and coupling procedures of different types, we found that porcine kidney aminoacylase is immobilized most successfully on solid supports with carboxyl functional groups, activated by water-soluble carbodiimides. By optimizing reaction conditions (enzyme : solid support : carbodiimide ratio, pH, temperature, ionic milieu), we succeeded in preparing immobilized aminoacylase with an activity relative to dry weight higher than that of any immobilized porcine kidney aminoacylase preparation described earlier (Ohno, Stahmann, 1971; Masková et al., 1973; Marconi, 1974). The activity of our preparation is higher than that of aminoacylases of microbial origin immobilized by various methods for industrial purposes (Kirimura, Yoshida, 1966; Tosa et al., 1967a, b, 1969a, b; Sato et al., 1971; Mori et al., 1972; Yokote et al., 1975).

The specific activity relative to protein amount of immobilized aminoacylase amounts to only ca. 25–30% of that of the soluble enzyme, indicating that a considerable fraction of the immobilized enzyme molecules is more or less inactivated. This, on the one hand, may be attributed to steric effects, e.g. covering of the active center by some structural part of the solid support or by the neighboring enzyme molecules, or binding via several amino acid side chains. On the other hand, inactivation may be due to the unsatisfactory selectiveness of coupling by carbodiimides, which may result in the modification of side chains essential for the maintenance of native structure.

Both its high activity and the excellent permeability of the bead polymer solid support renders our immobilized aminoacylase preparation suitable for practical utilization. This is also demonstrated by our successful experiments on the optimal resolution of DL-alanine, DL-methionine and DL-valine. Since the enzyme used in a column loses only 50% of its activity in ca. 2 months, continuous operation is easily realizable. The yield of the procedure (Table 4) is sim-

ilar to that obtained with other immobilized aminoacylase preparations described in the literature (Marconi, 1974; Yokote et al., 1975).

The authors are greatly indebted to Mrs Erzsébet Dala and Mr István Csereklye for their excellent technical assistance and to the Quality Control Section of Reanal for the accomplishment of certain analytical measurements.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 15 (4), pp. 305–311 (1980)

Changes in the Aerobic and Anaerobic Metabolism of Skeletal Muscle Subjected to Plaster Cast Immobilization

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(Received November 19, 1979)

The effect of immobilization for different periods (7, 14, 28 and 42 days) on the aerobic and anaerobic metabolism of rabbit muscles with different functions was studied by measuring the total activity of LDH and MDH as well as the distribution of their isoenzymes.

The results showed that on plaster casting the characteristic aerobic metabolism of m. soleus declined with a concomitant increase in glycolytic processes. In m. gastrocnemius the anaerobic metabolism of the muscle was relatively decreased on immobilization, but glycolysis remained the prevailing energy yielding process. On immobilization the metabolism of m. soleus and that of m. gastrocnemius approached each other. This may indicate a certain dedifferentiation of the muscle tissue.

Introduction

The atrophy of actively working muscles resulting from different damaging effects has been a problem of the clinical practice for a long time. Of the damaging effects, primarily genetic, toxic myopathies, tenotomic and neurogenic atrophies have been investigated morphologically and enzymatically (Dolan et al., 1975; Józsa et al., 1975; Knowltan Hines, 1934; Pette, Dölken, 1975; Usatenko, 1977).

As for the muscular alterations developing after plaster cast immobilization considerably less data are available. Traumatological observations have shown that muscles tolerate long lasting limb fixations in a very different manner. All such dissimilarities can be interpreted in terms of the different functions and metabolism of skeletal muscles. According to the accepted nomenclature in the literature fast glycolytic, and slow-oxidative fibres can be distinguished. Of the changes occurring in response to immobilization Guba et al. (1977) investigated the ultrastructural events, Takács et al. (1977) the distribution patterns of the sarcoplasmic proteins and Sohár et al. (manuscript submitted) the changes in the activities of some metabolic enzymes. All these biochemical parameters pointed to a certain dedifferentiation of the different muscles. Dedifferentiation was indicated by a decrease of the characteristic differences between the two types of muscle. Thus, it seemed worth to investigate the extent to which the aerobic–anaerobic metabolism of the muscles of different functions changes on plaster cast immobilization.

The extent of aerobic metabolism in tissues can be estimated enzymologically by the distribution of the isoenzymes of lactate dehydrogenase, LDH (EC 1.1.1.27) and malate dehydrogenase, MDH (EC 1.1.1.37). We studied the distribution of the isoenzymes of LDH and MDH of the muscles with different (oxidative and glycolytic) metabolism in the course of plaster cast immobilization.

Materials and methods

The experiments were performed with male New Zealand rabbits with an average weight of 2500 *g*. The right hind limbs of the 12-week-old rabbits were fixed in extension with circular plaster casting (Szöőr et al., 1977). Eight to ten animals were used in each group. After 1, 2, 4 and 6 weeks of fixing the plaster was removed, the animals exsanguinated and the m. soleus and m. gastrocnemius of the prepared limb were excised. M. soleus, containing mainly slow oxidative fibres was regarded as muscle with aerobic metabolism and the medial and lateral heads of the m. gastrocnemius as fast glycolytic muscles. Rabbits of the same breed, weight and sex served as controls. The excised muscles were freed from fat and connective tissue and weighed. Homogenization was performed in 20 volumes of 50 mM phosphate buffer (pH 7.2) containing 5 mM EDTA and 0.1 per cent Triton X-100 (Bass et al., 1969). After centrifugation (16,000 *g*, 15 min, 0 °C) the supernatant was used for further work. Protein content was determined by the microbiuret method (Goa, 1956).

Enzyme activities were measured by following spectrophotometrically the decrease of the concentration of NADH converted in the course of the reaction (Wroblensky, La Due, 1955). When measuring total LDH activity the substrate concentration was chosen such that the LDH isoenzymes with different subunit compositions took part in the reaction with the same activity (Schweitzer et al., 1973).

Total MDH activity was determined according to Bergmeyer (1974). Activities were measured at 25 °C, 340 nm using a UV-VIS type spectrophotometer.

Separation of LDH and MDH isoenzymes was performed by gel electrophoresis using 5.5 per cent polyacrylamide gel. Enzyme activities were visualized by tetrazolium-blue staining (Dietz, Lubrano, 1967) using the appropriate substrates. The intensity of the individual bands was assessed by a Kipp and Zonen densitometer.

Statistical evaluation of the results was made by Student's *t* test. Values with $p < 0.05$ were regarded as statistically significant.

Results

With increasing time of immobilization the total activities of both LDH and MDH gradually decreased (Table 1). The extent of decrease of enzyme activities, however, was different in the two metabolically distinct muscles. In m. soleus

Table 1

Changes in the total activity of LDH and MDH and in the LDH/MDH ratio during immobilization

Time of immobilization (weeks)	M. soleus			M. gastrocnemius		
	LDH	MDH	LDH/MDH	LDH	MDH	LDH/MDH
0	2.9 ± 0.5	1.1 ± 0.16	2.6	10.7 ± 1.8	1.0 ± 0.15	10.7
1	2.3 ± 0.4	0.8 ± 0.23	2.9	10.1 ± 3.7	0.9 ± 0.06	11.2
2	1.7 ± 0.2*	0.5 ± 0.08*	3.4	7.8 ± 0.8*	0.9 ± 0.15	8.7
4	1.1 ± 0.3*	0.3 ± 0.07*	3.7	3.7 ± 0.7*	0.8 ± 0.07*	4.6
6	1.3 ± 0.5*	0.2 ± 0.01*	6.5	3.2 ± 0.7*	0.7 ½ 0.01*	4.6

Activities are expressed as U/mg protein
* significant change ($p < 0.05$)

the rate of reduction of the activity of MDH considerably exceeded that of LDH. The LDH/MDH ratio grew from 2.6 (oxidative type) to 6.5 on the 42nd day of immobilization. Changes in m. gastrocnemius, in turn, showed an opposite tendency. Here, the LDH/MDH ratio greatly decreased from an initial value of 10.7 (glycolytic type) to 4.6 on the 42nd day.

The per cent distribution of the LDH isoenzymes (Fig. 1) well demonstrated the oxidative metabolism of m. soleus in the control animals. The dominance of isoenzymes LDH-1 and LDH-2 is characteristic. With increasing time of immobil-

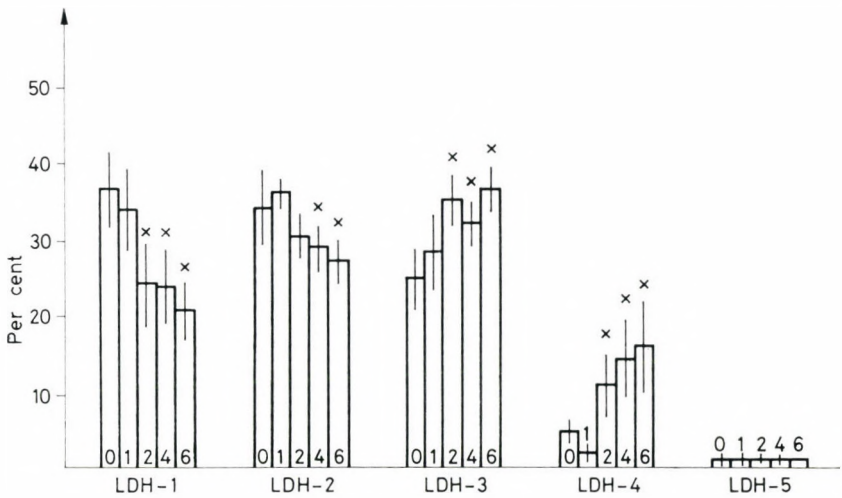

Fig. 1. Distribution of LDH isoenzymes in m. soleus. Figures in the columns indicate the time of immobilization in weeks. Zero stands for the control. Mark "x" denotes changes at a high level of significance ($p < 0.05$) as compared to the control

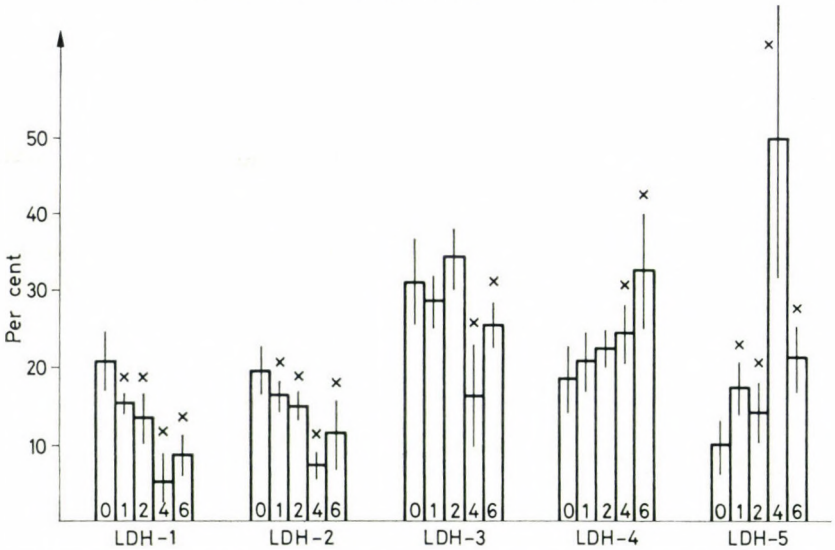

Fig. 2. Distribution of LDH isoenzymes in m. gastrocnemius. The markings are the same as in Fig. 1

ization the amounts of LDH-1 and LDH-2 diminished while those of LDH-3 and LDH-4 gradually increased. Fraction LDH-5 could not be detected in the m. soleus of either the immobilized or the control animals.

In the m. gastrocnemius of control animals, in accordance with its glycolytic metabolism, isoenzymes LDH-3, LDH-4 and LDH-5 predominated as compared to m. soleus (Fig. 2). In response to the immobilization the relative amounts of LDH-4 and LDH-5 further increased while the quantities of LDH-1 and LDH-2 decreased.

The ratios of the subunits (H and M) constituting the LDH isoenzymes were also calculated. The value of the H/M ratio was characteristically high in tissues with aerobic metabolism and low in the glycolytic muscle that produces lactic acid in abundance (Pfleiderer, Wachsmuth, 1961). Table 2 shows that upon

Table 2

Changes in the H/M and mMDH/sMDH ratios during immobilization

Time of immobilization (weeks)	M. soleus		M. gastrocnemius	
	H/M ratio	mMDH/sMDH ratio	H/M ratio	mMDH/sMDH ratio
0	3.3	0.63	1.3	0.45
1	3.0	0.61	0.9	0.31
2	2.0	0.54	0.9	0.35
4	1.9	0.52	0.4	0.44
6	1.7	0.56	0.5	0.42

immobilization this ratio greatly decreased for both m. soleus and m. gastrocnemius.

We also investigated the distribution of MDH isoenzymes in the mitochondria (mMDH) and in the cytoplasm (sMDH) and calculated the mMDH/sMDH ratios for both muscles. As expected, the value of the above ratio was higher in m. soleus. During immobilization this ratio gradually decreased in the case of m. soleus, while for m. gastrocnemius, after an initial decline, on day 28 the ratio again approached the value of the distribution found in the normal muscle.

Discussion

The distribution of enzymes in different types of skeletal muscle varies according to the function of the muscle (Bass et al., 1969, Pette, Dölken, 1975) and undergoes characteristic alterations on training (Apor, 1977; Vihko et al., 1978) or following muscle damage (Usatenko, 1977; Mazarean et al., 1979). In previous reports we have demonstrated that on plaster casting the weights of m. soleus and m. gastrocnemius of the rabbit decrease (Szőőr et al., 1977) and both the quantitative and qualitative parameters of the sarcoplasmic proteins change. In addition immobilization modifies also the balance of aerobic and anaerobic processes in the muscles with different functions.

According to our results, in the slow-oxidative m. soleus the changes in the LDH and MDH activities unequivocally show a decrease of the intensity of aerobic processes and a relative preponderance of the glycolytic metabolism. Increase in the LDH/MDH ratio and accumulation of M subunits in the LDH isoenzyme spectrum also support the above finding. The prominence of the cytoplasmic, glycolytic processes is also indicated by a relative increase in the amount of sMDH.

Similar changes have been reported by Dolan et al. (1975) in m. soleus on denervation and demyelinization. Their experiments on mouse showed that the H/M ratio invariably decreased to one-third of its original value on both types of intervention. The authors emphasized the role of neuronal trophic effects in securing the metabolism of the muscle. Since no change has occurred in the innervation of the muscle on immobilization, the alterations in m. soleus we observed cannot be interpreted in terms of a cessation of tropic effects.

In the m. gastrocnemius the decrease of the total LDH activity indicates a slowing down of glycolytic processes on plaster casting. Oxidative metabolism is less affected as indicated by the decrease of the LDH/MDH ratio. All these, however, do not mean a qualitative change in the metabolic pattern of m. gastrocnemius. Although anaerobic metabolism, characteristic of this type of muscle, decreased to a relatively larger extent than aerobic metabolism did, glycolysis was maintained as the major energy-yielding process. This is also supported by the accumulation of the M subunits, a change that can be considered as a compensating alteration. The muscle responds to the new metabolic design brought about

by a decreased total activity of LDH, by an increase in the relative amounts of LDH-4 and LDH-5, characteristic of anaerobic metabolism (Pfleiderer, Wachsmuth, 1961). Thus, the metabolism of the two muscles in question changes on plaster casting. In the soleus the anaerobic processes will prevail while in the gastrocnemius, although to a lesser extent, the dominance of the glycolytic processes will be preserved.

In the pathogenesis of muscle atrophy, brought about by immobilization, presumably several important factors are involved. In our opinion, diminished tissue blood circulation due to the deletion of active muscle function (Solti, 1977) is an important inducing factor. The resulting tissue hypoxia, subsequent metabolic switches and shifts in substrate concentrations may give rise to regulatory changes in the rates of synthesis of individual enzymes. On the other hand, the more intensive catabolism induced by immobilization (Goldspink, 1977; Jakubiec-Puka, Drabikowski, 1978) may alter the catabolic rate of the different enzymes and shift their half life. Presumably, as a result of both processes, the metabolic difference between m. soleus and gastrocnemius decreases and a certain dedifferentiation takes place. On immobilization, mainly as a result of changes in m. soleus, the metabolism of m. soleus and that of m. gastrocnemius will approach each other.

The authors are grateful to Dr Teréz Szabó for her help in measuring MDH activities, as well as to Miss Anna Bogdán and to Mrs István Varga for their useful technical assistance.

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Book Reviews

Current Topics in Microbiology and Immunology Vol. 86, by W. Arber et al., (eds) Springer-Verlag, Berlin–Heidelberg–New York 1979, p. 168, with 29 Figs.

Volume 86 of this popular series comprises four chapters: two on defective interfering viruses, one on Bunyaviruses and one on the FV-1 gene of the mouse.

Recent developments in the molecular biology of a fairly new group of viruses are summarized by D. H. L. Bishop in the *Genetic potential of Bunyaviruses*. More than two hundred arthropod-borne viruses are now registered, or categorized as possibly belonging to the family Bunyaviridae. The distinguishing features of Bunyaviruses are the following: (i) They have a three segment, single-stranded, negative-sense RNA genome with a total molecular weight of $4-6\times10^6$ daltons, (ii) the virus particles are enveloped and spherical (about 100 nm in diameter), (iii) virus-specific glycoproteins are located in the outer surface of the envelop, and (iiii) replication takes place in the cytoplasm and particles are formed by budding into Golgi cisternae. Bunyaviruses are divided into eleven serogroups although by comparison with other enveloped viruses it has not been determined which viral polypeptides are involved in the different serological tests. Three major structural polypeptides have been identified: two external glycoproteins (G_1 and G_2) and one internal nucleoprotein (N) with molecular weights of 115×10^3, 38×10^3 and 19×10^3 daltons, respectively. Three RNA species of the genome, large (L), medium (M) and small (S), with molecular weights of 3, 2 and 0.5 million daltons, respectively are found in circular, helical nucleocapsids. Genetics analysis has been performed with conditionally-lethal temperature sensitive mutants. It has been established that segment S codes for polypeptide N, segment M for G_1 and G_2, while L codes for a virion transcriptase. It has also been demonstrated that recombination takes place by segment reassortment.

At the time of their discovery, the problem, of defective interfering (DI)-particles seemed to be a "by-product" of autointerference and interferon research. Now it is an independent discipline of virology with the possibility of gaining insight into the mechanism of virus multiplication. This is also substantiated by the reviews on *DI-particles of togaviruses* (by V. Stollar) and *DI-particles of rhabdoviruses* (by M. E. Reichmann and W. M. Schnitzlein). DI-particles contain deleted viral genome, but a complete set of structural proteins. They require homologous "healthy" (standard) virions for replication, but interfere specially with them. This interference requires that DI-particles possess a functional nucleic acid piece. The deleted RNA represents 20—30% of the genome with sequences overlapping both or just one of the ends of the genomic RNA. In the case of rhabdoviruses having a negative-sense RNA, most DI-particles are the subsets of the 5'-end of the genome, thus overlapping a part of the transcriptase gene. According to models, during viral infection de novo generation of DI-particles may result in a decreased amount of viral progeny. In tissue cultures a cyclic pattern of virion and DI-particle production is maintained in the presence of limited amounts of viral antiserum suggesting that recurring viraemia could take place in the animal in the presence of reduced levels

of antibodies, that is when immunity is not adequate. The central question at the moment is whether DI particles can alter the course of a natural viral infection.

The FV-1 gene of the mouse and its control of murine leukemia virus replication (by P. Joliceur) provides an up-to-date account of this complex and intricate host–virus system. Fv-1, the gene controlling the susceptibility of mouse cells to Friend virus (a laboratory-derived oncogenic virus) was discovered only a decade ago. Susceptibility to naturally occurring leukemia viruses (MuLV) is also governed by the Fv-1 locus which is equally expressed in the animal and cultured cells suggesting a purely cellular mechanism of action. All the MuLV strains fell into one of two categories with respect to their host range, being either N- or B-tropic. Since all the mouse strains are also N- or B-type, this highly conserved complementarity suggests a very close virus–cell interaction during evolution. Fv-1 acts not by increasing susceptibility to a given virus, but by restricting the growth of viruses of the opposite tropism. Fv-1 gene is mapped on mouse chromosome 4. There are several factors (X rays, steroid hormones, etc.) which can overcome the restriction of virus multiplicatiom governed by the Fv-1 gene. It seems that a specific gene product is responsible for the Fv-1 action. Other relevant topics such as conversion of viral tropism, viral determinants of tropism, biology of N- and B-tropic viruses, leukemiogenicity are also discussed.

B. Lomniczi

Current Topics in Microbiology and Immunology. Vol. 80, by Arber et al. Springer-Verlag, Berlin—Heidelberg—New York 1978. p. 169.

This volume deals with five very diverse topics which, however, have in common that they all have some bearing on immunology.

The first review, "Role of Iron in Bacterial Infection" by J. J. Bullen et al., describes the properties of iron-binding proteins with special reference to the role of transferrin in iron transport and that of lactoferrin in hypoferremia, the lowering of serum iron levels in inflammatory processes. A separate chapter of this review is devoted to iron-binding proteins and resistance to infection by *Yersinia pestis*, *Pasteurella septica*, *Clostridium welchii* Type A, *Pseudomonas aeruginosa* and *Escherichia coli*. The protective effect of colostrum and milk is given special emphasis. Next the role of iron-binding proteins in polymorphonuclear cells have recently been recognized to play an important role in the destruction of bacteria. Iron-binding proteins, whether intra- or extracellular, contribute to resistance against bacterial infections. Fe saturation of both extracellular and phagocytic systems is known to influence the disease resistance of organism to a considerable extent.

The introductory chapter of the second review, "Effective and Ineffective Immune Responses to Parasites: Evidence from Experimental Models" by J. H. L. Playfair, gives a tabular survey of host–parasite relationship: six levels of associations are distinguished. A short characterization of natural immunity is followed by a much longer chapter on adaptive immunity in which cytotoxicity and dytostasis, antibody formation and regulatory cells are dealt with. The next and actually longest chapter is about malaria. The following major topics are discussed: immune responses in malaria, the role of T cells (including the helper T cells, delayed hypersentivity, cytotoxicity and suppressor T cells), antibody and malaria, non-specific immunostimulation, and evasion mechanisms. The description of antigenic variation in malaria is of special interest. The next chapter deals with trypanosomiasis, with special reference to antigenic variation, immunosuppression and soluble complexes. Additional chapters are about toxoplasmosis and leishmaniasis, including the role of T cells and possibilities of vaccination. In the chapter on schistosomiasis, caused by the most important human trematode, the possibility of vaccination with irradiated vaccines is discussed. The chapter on the role of both specific and non-specific IgE in worm infections is especially stimulating. In the author's view it would be interesting to study the effect of manipulating the IgE/IgG balance on the course of these diseases. In the Conclusion the author warns against making simple generalizations about immunity to parasites, because the mechanis, of the

immune response varies to a considerable extent with individual parasites. In some cases it is the trend of adaptation of the immune system, in others the amount of the chemical mediator produced by the T cells that determines the intensity of the defence reaction. On the whole, this review is a very valuable summary of our present knowledge of parasitic diseases which are known to involve at least two thirds of the world's population and yet are studied to a lesser degree than they should be.

The third review, "In Vitro and in Vivo Investigations on Antibody-Dependent Cellular Cytotoxicity" by G. R. Pearson, first deals with the methodology for demonstrating antibody-dependent cellular cytotoxicity (chromium-51 release assay, microcytotoxicity test and plaque assay). This is followed by a discussion of the nature of antibodies capable of mediating antibody-dependent cellular cytotoxicity. In the chapter about effector cell populations animal and human systems are treated separately. In the next two chapters we learn about the mechanism of target cell destruction and antibody-dependent cellular cytotoxicity against virus infected cells, respectively. Application of antibody-dependent cellular cytotoxicity to human diseases covers cancer, transplantation immunity and autoimmune diseases. As put forward in the Summary, the nature of the major lymphatic effector cell in antibody-dependent cell-mediated cytotoxicity seems to be a cell that lacks both T- and B-cell characteristics but bears the receptor for the Fc fragment of the immunoglobulin molecule. This cell seems to be the same in both human and animal systems. Other cells can also participate in this cytotoxic reaction, depending mainly on the nature of the target cell, but the lymphatic killer cell is of great importance.

The fourth review is by S. Cohen and G. H. Mitchell and deals with prospects for immunization against malaria. After summarizing the geographical distribution and transmission of this parasitic disease, the authors review the life cycle of the parasite and the etiology of the disease with special emphasis on erythrocytic infection. The remaining major part of the review deals with vaccination. This material is divided into two major chapters: one about vaccination against the pre-erythrocytic stage and the other one about vaccination against the erythrocytic stage. In the former one questions related to the immune response to live unattenuated sporozoites, inactivated sporozoites, irradiated sporozoites, specificity of sporozoite-induced immunity, mechanism of sporozoite-induced immunity and vaccination with exo-erythrocytic merozoites are discussed. The chapter on vaccination against the erythrocytic stage provides detailed discussions of immunization by controlled infection, irradiated parasitized red cells, attenuated blood parasites, fractions of parasitized erythrocytes, killed parasitized red cells, blood-stage merozoites, specificity of immunity against the erythrocytic stage, adjuvants in vaccination against blood stage parasites and mechanisms of immunity to the erythrocytic stage of malaria. A short chapter on vaccination with gametes is followed by an evaluation of methods of malaria vaccination. The review concludes with a chapter on the clinical trial of a human malaria vaccine. The authors conclude that at the present time blood-stage merizoites constitute the most promising form of vaccine for potential use in human trials. Their practical advice on the proper timing of vaccination is of special value.

The last review in this volume is by C. Scholtissek and gives a critical analysis of the genome of the influenza virus. The author sets the stage for subsequent chapters by giving a short historical background and by summarizing the basic principles of the nomenclature of influenza viruses, including serologic and epidemiological characteristics. The succeeding chapters follow a very logical order from subjects of a basic nature to topics of a more practical significance. First the structure and mode of multiplication of influenza virus are discussed with an emphasis on the identification of viral gene products. A deeper insight into the genetics of influenza viruses is successively set forth by individual chapters on temperature-sensitive mutants of influenza viruses with special reference to the number of recombination/complementation groups, on the structure of the viral RNA, on reassortment and formation of recombinants, on assignment of RNA segments to viral gene functions and/or proteins, and on base-sequence homo-

logies between RNA segments of different prototype strains. These chapters provide a firm basis for the understanding of medical problems reviewed in the chapters on gene constellation and pathogenicity, on the potential use of recombinants and temperature-sensitive mutants for vaccines, and on the abnormal genetic behavior of influenza viruses. In his concluding remarks the author points out that from a medical point of view influenza is still an unresolved problem, mainly due to the unusual antigenic variability of the causative agent. This is reflected in the sudden occurrence of new subtypes. This peculiar behavior of the virus is based on its segmented genom. Each RNA segment (there are eight of them) codes for a single polypeptide. New recombinants may easily evolve by double infection of a common host. Such new strains would not find protective immunity in man. The author concludes his review by calling our attention to the practical significance of "synthesizing" new recombinants that can be used as live vaccines and to the theoretical importance of determining the complete sequence of all eight RNA segments of influenza viruses.

F. ANTONI

Current Topics in Microbiology and Immunology, Vol. 87, by W. Arber et al. (eds), Springer-Verlag, Berlin–Heidelberg–New York 1979, p. 172, with 24 Figs.

Graessmann, A., Graessmann, M. and *Müller, C.:* Simian virus 40 and polyoma virus gene expression explored by the microinjection technique. Injection of virus, viral DNA, viral RNA or T antigen into permissive and non-permissive cells proved to be a useful method for studying regulatory mechanisms responsible for the mode of viral gene expression in different types of infection. It has been concluded that induction of viral DNA replication and late viral gene expression are highly dependent on the quality of T antigens synthesized. Resistance is determined by an early event preceding virus uncoating while in permissive cells viral DNA syntesis mediates late viral gene expression and determines the fate of infected cells. As for the technique, 1000 cells per hour can be injected.

Taylor, J. M.: DNA intermediates of avian RNA tumor viruses. During retrovirus infection various DNA intermediates emerge as a result of reverse transciption. One of these is invalued in the normal cycle of virus infection. At least two conformations of unintegrated DNA intermediate can be found in infected cells: a) a linear double-stranded intermediate that appears to be a uniform transcript of viral RNA into double-stranded DNA; b) covalently closed circular DNA intermediates found in the nucleus. The former is found in the cytoplasm and could by synthesized in vitro as well. Both intermediates are infectious, which also means that both are capable of integration into cellular DNA. The site on cellular DNA where integration takes place seems to be nonspecific. Recently a 12-nucleotide sequence has been observed on the viral DNA that may correspond to a potential promoter for RNA polymerase II. This sequence is repeated at a location about 120 nucleotides upstream from the regular promoter site, which explains the great efficency of retrovirus transcription in infected cells: one integrated provirus may produce 20,000 RNA transcripts per cell.

Lebowitz, P. and *Weissman, S. M.:* Organization and transcription of the Simian virus 40 genome. The entire nucleotide sequence has recently been established, making it possible for the first time to localize the templates of the known viral mRNAs and proteins. However, many old problems have remained unsolved and some new questions arose, such as: the genome of SV40, the proteins of SV40, templates for viral proteins and functions, viral transcription during lytic infection, genome organization and transcription in cells transformed by SV40, and genomic organization and transcription in adenovirus–SV40 hybrids.

B. LOMNICZI

Tumor Antigenicity and Approaches to Tumor Immunotherapy by David W. Weiss, Current Topics in Microbiology and Immunology, Vol. 89. Springer-Verlag, Berlin–Heidelberg–New York, 1980.

This volume is concerned with the most important approaches to immunological intervention in cancer, in the light of current

information on distinguishing antigenicity of neoplastic cells.

Of the several distinct avenues of immunotherapy, active "non-specific" immunotherapy with immunomodulating agents is analyzed in detail. Emphasis is layed on microbial agents that have shown so far, on the whole, the greatest efficacy in experimental animal test systems and have attracted the widest clinical attention: the MER fraction of tubercle bacilli, which has been a subject of the writer's interest for many years. The possible advantages inherent in the non-specific approach to tumor immunotherapy is summarized bearing in mind that therapeutic immunomodulation is a two-edged sword and also that we have to overcome the disparity in results obtained with such modulatory therapy in animal and human neoplasias.

The next part deals with the second major pathway in immunotherapy, the active specific immunization with tumor cell antigens. Not only activation of cellular immunological elements, but also improved antibody formation are commented upon. Active immunization might have some role in tumor prophylaxis.

Passive (adoptive) immunological intervention is outlined as the third main trend in cancer immunotherapy. This approach has undeniable advantages, especially with regard to the employment of cytotoxic effector cells and antibodies that are produced in tissue culture. Potentiation of cytotoxic activity of in vitro educated effector cells, both human and murine, is also mentioned. A critical view on possibilities and problems of clinical application is provided.

Before the concluding remarks Weiss emphasizes that immunological intervention in neoplastic diseases of man is an exceedingly complicated task of "bioengineering", requiring a sophisticated apprehension of immunological function and of host–tumor interactions still far beyond our reach. The experimental models, although unable to provide direct and detailed formulas for the clinicians, are regarded as stepping-stones toward intelligent clinical inquiry. Certainly, only clinical testing will disclose what benefits can be achieved by this modality, and the application in the clinic will have to be made with large measures of cautious empiricism for all the encouragement from animal experiments.

The concluding remarks focus on key aspects, goals and expectations of immunotherapy, experimental perspectives, immunological capacity and monitoring. It seems to be essential to mention that surveying the major avenues of immunotherapy Weiss emphasizes the importance of combined modality treatment of human cancers and also the finding of guiding criteria to apply precisely what appears mandatory for optimal immunotherapy.

This book provides a critical treatment of a rapidly changing field, tumor immunotherapy, with selected references to the current and past literature. It serves the interest of all scientists involved in clinical or experimental oncology.

L. KOPPER

Medicinal Chemistry, by M. J. S. Dewar, K. Hafner, E. Heilbronner, S. Ito, J.-M. Lehn, K. Niedenzu, K. Schäfer, G. Wittig (eds).*Topics in Current Chemistry*, 72. Managing Editor: F. L. Boschke. Springer-Verlag, Berlin–Heidelberg–New York 1977.

The volume consists of 5 parts

1. F. E. Hahn: Modes of Action of Antimicrobial Agents,
2. W. Wehrli: Ansamycins,
3. D. Orth, H.-E. Radunz: Heteroprostanoids,
4. E. Schacht: Hypolipidaemic Aryloxyacetic Acids,
5. P. Chandra, G. J. Wright: Tilorone Hydrochloride.

The first part of the book (Modes of Action of Antimicrobial Agents) describes on 19 pages the mode of action of antimicrobial agents on the basis of an enormous work which had been published in more than 10,000 original papers. First, the author refers to his own lecture on the concepts of inhibited reaction, inhibition and degree of inhibition. Then, he reviews the basic papers on the mechanism of action of chloramphenicol, chlortetracycline, some antimalarials, etc. Mainly *Escherichia coli* was chosen as general test organism for antibacterial agents, with reasonably broad spectra, but sometimes *Mycobacteria*, *Bacillus megaterium*, etc. were also used to study drug effects. The

strategy of studying the mode of action of these drugs has to take into account the fact that there are just a few points of interference with physiological processes of microbial cells that result in cell death or inhibition of cell replication. These are the following: 1. Inhibition of DNA synthesis, 2. Inhibition of RNA synthesis, 3. Inhibition of protein synthesis, 4. Inhibition of cell wall synthesis, and 5. Interference with the structural and functional integrity of cell membranes. Studies of these topics are reviewed, and most interesting photograph (under fluorescence microscope, phase contrast microscope, etc.) as well as very representative diagrams of selective inhibitions are given.

The second part (Ansamycins, Chemistry, Biosynthesis and Biological Activity) is divided according to the decimal system. The first chapter deals with the chemistry of ansamycins, namely rifamycins, streptovaricins, tolypomycin Y, naphtomycin, geldanamycin, streptolydigin, tirandamycin, maytansin and related compounds. The very well selected figures show the schematic structure of ansamycins, the stereomodel of rifamycin SV as well as the chemical structures of the above mentioned members of the ansamycin family. The biosynthesis, origin and biological activity of ansamycins are detailed in the second chapter, the understanding of the topics is facilitated by figures. The third chapter seems to be the most detailed and impressive one, in which the various modes of action are described. Special attention is given to their effect on bacteria, eukariotes, RNA tumor viruses, DNA tumor viruses and larger infectious agents. Conclusions and a summary close this well written part.

In the third part of the book (Heteroprostanoids) the authors review the prostaglandin-like compounds that contain one or more heteroatoms in the prostanoic acid skeleton in place of one or more carbon atoms. The synthesis of various thioprostanoids, oxaprostanoids, azaprostanoids as well as dihetero- and trihetero-prostanoids is presented. The preparation of these compounds is described in it most detail as far as the extent of the chapter allows it. The activity of heteroprostanoids is presented in Table 1. The up-to-date and full reference list comprises 127 published works.

The fourth part of the volume (Hypolipidaemic Aryloxyacetic Acids) also deals with an interesting topic. In the introduction the author outlines the site of the "increase of plasma lipids" which is one of the so called "risk factors" of arteriosclerosis. At the same time, hyperlipoproteinaemia is recognized to be at least an additional factor involved in the pathogenesis of the following disorders and diseases; hyperuricaemia, hyperinsulinaemia, gallstones, pancreatitis, skin lesions, decreased glucose tolerance, fatty liver. Although the correlations between the increased blood lipids and the above mentioned diseases are mainly unknown, the lipid lowering therapy and its tools can give the starting point of the physiological, pathophysiological and therapeutical investigations. The author gives numerous data about the substituted aryloxyacetic acids in Chapter B, and the chemistry and physicochemical characteristics of these compounds in Chapter C, mainly in tables. The discussion chapter deals with the effect of some of these compounds on the lipid level. The results are really interesting but their perspicuity could have been facilitated at least by their arrangement according to the decimal system or in a tabular form. Despite this shortcoming, the valuable data and knowledge presented here form a really necessary complement to the literature of hypolipidaemic compounds.

The fifth paper of the book (Tilorone Hydrochloride) is an overview of the chemistry and different effects of tilorone and related compounds. Tilorone was found to be a broad spectrum antiviral agent which could be administered orally (in mice). Its antitumor activity, anti-inflammatory properties and influence on immune mechanisms are also described. The chemistry, toxicological evaluation, disposition and pharmacokinetics of tilorone are described in detail on the basis of several publications. Especially in connection with this latter topic modern methods are mentioned, such as thin-layer chromatography and detection of ^{14}C-tilorone and its metabolites. The effect of tilorone on the drug metabolizing enzymes of the rat liver, its antiviral and interferon inducing effects as well as the interaction of tilorone hydrochloride with some macromolecules are also reported. This

latter is clearly demonstrated by a change in the absorbtion spectrum in visible light and the results are confirmed by other modern methods. Suggestions concerning some future prospects, an addendum and references conclude this part.

The volume is competed with an author index to volumes 26—72.

The 72nd volume of Topics in Current Chemistry will certainly be very useful to all researchers who work in any of the fields treated in this book.

H. KALÁSZ

Biochemistry. Topics in Current Chemistry Vol. 78. (Fortschritte der Chemischen Forschung) by Boschke, F. L. (ed.), Springer-Verlag, Berlin—Heidelberg—New York 1979, p. 193, 32 Figs, 23 Tables.

The volume consists of three main chapters. The chapters are subdivided according to the decimal system, which makes the contents comprehensive and well arranged.

W. Hasselbach is the author of the first main chapter entitled "The Sarcoplasmic Calcium Pump. A Model of Energy Transduction in Biological Membranes", which is divided into 6 subchapters. The introduction gives an overview of the ion pump, the action of the active sodium pump on cellular equilibrium processes as well as the regulation of some cellular activities and the identification of the energy source of active ion movement. The references are taken far back from around 1940 and the very recent data are also cited in a good selection. The second subchapter gives a valuable characterization of the sarcoplasmic reticulum membranes and their calcium pump is visualized by electron micrographs of sarcoplasmic reticulum and its fragmented constituents. The lipids and proteins of the sarcoplasmic reticulum are set forth in three tables and the separation of protein constituents and their triptic fragments is presented in two figures. The electron micrographs and the separation of native proteins in SDS-containing solutions by PAGE are especially demonstrative and impressive. The 3rd and 4th subchapters on "Energy Dependent Calcium Translocation" and "Functional Aspects of Lipid–Protein Interaction", respectively, are well fitted together and the diagrams demonstrate the content brilliantly. The 5th subchapter deals with substrate and reaction intermediates. This part is also well documented with figures and tables but the discussion of binding as well as that of phosphorylation would have needed at least the mentioning of the methods applied in addition to the references.

G. Krampitz and W. Witt review the "Biochemical Aspects of Biomineralization" which is the second main chapter of the book. As they emphasize in their introduction, biomineralization can be considered as a very wide-spread phenomeneon as far as the formation of bones, teeth and shell's material, characteristic of the majority of approximately 128,000 species of molluscs, is concerned. The authors examine the inorganic and organic components of biomineralization in their second subchapter and the main roles of the constituents are also mentioned. It is probably the enormous amount of the data that impelled the authors to refrain from summarizing their material in a tabulated form as it had been done in other reviews of the same topic. Anyhow, the interpretability of this chapter would have been basically increased by supplying more numerical data. The very impressive Figs. 1/a and 1/b do not mention the rate of magnification. The 3rd and 4th subchapters (Cellular Aspects of Biomineralization and Mechanisms of Calcification) are more informative and the essence of their topics is presented in simple but informative figures and schemes. The latter two subchapters can particularly be recommended to gain novel and comprehensive informations. The up-to-date reference list with 643 cited works completes this chapter.

M. Nozaki's work is the third main chapter entitled "Oxigenase and Dioxigenase". The detailed discussion of these two enzymes is mainly based on well constructed tables and a relatively short text. Formulas and reaction schemes support and explain the valuable informations. Subchapter I is the introduction, subchapter II gives the classification of the oxigenases. Subchapters III and VI give a review of nonhem iron-containing-, heme-containing-, copper-containing- and flavin-containing oxigenases. Subchapter VII deals with the description of dioxigenase involved in the formation of

catechol derivatives and Subchapter VIII with the -ketoglutarate-requiring dioxygenase. A short but informative summary ("Concluding Remarks") is also part of the final subchapter and gives a perfect overview of oxygenases and dioxygenases. The chapter concludes with a reference list containing 226 cited works. The whole chapter is really informative and gives a number of particulars in tables and figures, which are in good agreement with the text. There is a wide spectrum of applied and cited methods from gel chromatography to Mössbauer spectroscopy. The reference list also mirrors the compactness of the chapter with well selected citations.

The 78 the volume of the series "Topics in Current Chemistry" gives practically full information about the essence of three important topics of biochemistry, namely the sarcoplasmic calcium pump, the biochemical aspects of biomineralization and the oxygenases. Well set up tables and figures and more than 1000 references complete the three valuable chapters which are helpful for researchers in the fields treated in this volume.

H. KALÁSZ

Chemoreception in the Crotid Body by H. Acker, S. Fidone, D. Pallot, C. Eyzaguirre, D. W. Lübbers, R. W. Torrance, 296 pages with 101 figures and 21 tables, Springer-Verlag, Berlin—Heidelberg—New York 1977

The book comprises 40 papers presented by 52 participants of the International Workshop held at the Max-Planck-Institut für Systemphysiologie in Dortmund/FRG.

This workshop was the fourth international meeting of scientists interested in the research of the carotid body, the glomus caroticum which is regarded as an important peripheric organ of chemoreception. The first meeting on the same subject was held in Oxford in 1966, the second in Bristol in 1973 and the third in Kashmit in 1974. At the preceding meetings research was focussed on the cellular mechanisms of chemoreception. In line with this trend, the papers held at the workshop in Dortmund dealt mostly with the different cell types in the carotid body, their light and electron microscopy, measurement of their environment such as local PO_2 and blood flow, as well as with histochemical, biochemical and electrophysiological studies.

In accordance with the six sessions of the workshop, the papers were grouped in six chapters.

Session I: *Morphometric and histologic analysis of the cell elements in the carotid body tissue*

From this chapter we learn that the specific tissue (type I and type II cells) represents only a small portion (20%) of the carotid body tissue, the nerves contributing 60% and the vessels 20% to it in the cat. It seems rather difficult to identify the sensory nerve endings even by using isotopes or denervation, It was astonishing to learn that after having cut the carotid sinus nerve chemoreceptor responses were recorded from regenerating fibres at a time when synapses between regenerating fibres and specific cells in the carotid body were still missing. Axon terminals containing numerous mitochondria were interpreted as pressor-receptor endings in the mouse, cat and rat carotid body. The Discussion part shows that the morphological basis of the receptory function is not yet clear. In this chapter it is particularly interesting to have a stereoscopic view of the computer drawing of a glomoid, the supposed functional unit of the organ.

Session II: *Electrophysiological characteristics of the cell elements in the carotid body*

This short chapter consists of four papers only. They reveal information about effects of temperature, of various chemical stimuli and changes of osmolarity on type I cells in situ or maintained in tissue culture.

Session III: *Histochemical and biochemical investigation of the transmitters in the carotid body*

This chapter deals with the level of endogeneous acetylcholine, the distribution of labelled choline and changes in tyrosine hydroxylase activity elicited by hypoxia. During hypoxia the content of dopamine decreased without any change in the norepinephrine level. Decrease of the intensity of catecholamine fluorescence was also observed after inhibition of tyrosine hydroxylase

by alpha-methyl-para-tyrosine. Further, controversial data on the existence of dopamine-beta-hydroxylase activity were reported.

Session IV: *The afferent and efferent chemoreceptive pathway of the carotid body*

Here, we learn about various pharmacological effects on chemoreceptor discharges of the carotid sinus nerve. The sympathetic nervous system is reported to have variable influences on carotid body chemoreceptor activity. Studies on the fluctuation of chemoreceptor discharge in naturally breathing rats indicate that peripheral chemoreceptors do not contribute to respiratory drive. In one of the papers the primary projection of carotid chemoreceptors in the oblongate medulla was studied. The last paper gives a statistical analysis of the functional relationship between ventilation and arterial oxigen pressure.

Session V: *Morphometric analysis of ultrastructural changes in the carotid body tissue*

This is again a short chapter dealing with monoamine fluorescence of the carotid body cells and electron microscopic examination of the empty granular vesicles present in them. The chief cells of the glomus caroticum are supposed to be "paraneurons", cells being more or less similar to neurons and predominantly of secretory character.

Session VI: *Environmental conditions for the chemoreceptive process in the carotid body*

The topics of papers here are similar to those of chapter II: factors affecting O_2 consumption, analysis of oxigen partial pressure, measurement of tissue PO_2, role of calcium ions in chemoreception, effects of temperature on carotid chemo- and baroreception, finally a study on the role of bicarbonate in the carotid body.

Among the 40 papers presented in the book, only a few include a summary. It would have been better to publish them in standard form, each one with an informative synopsis. On the other hand, the discussions published are highly informative by pointing out the most interesting problems of the corresponding paper. To sum up my impressions about the book I think it gives a good survey of trends and problems in the research of the carotid body, it is useful for everybody interested in chemoreception, respiratory regulation and chromaffin tissue.

B. VIGH

Mathematical Models in Cell Biology and Cancer Chemotherapy by Martin Eisen. In: Lecture Notes in Biomathematics, Vol. 30. (S. Levin, ed.), Springer-Verlag, Berlin—Heidelberg—New York 1979, 431 pages, 71 Figures, 15 Tables, 581 equations, 491 references

The purpose of this monograph is to indicate how mathematicians can contribute to fighting cancer. That is why pharmacological knowledge concerning cancer chemotherapy is not required from the reader.

The major difficulty in chemotherapy is that most cancer drugs kill normal as well as tumor cells. Therefore drugs are usually given on a cyclic basis: periodic rest periods allow the patient's normal time to recover. It is well known that optimal scheduling can make the difference between success and failure in chemotherapy.

In this book some mathematical techniques are described for devising "optimal" schedules. Such an approach must have a sound biological basis. Hence, the book begins with a chapter on the structure, function and division of the cell, and contains appendices, such as chemistry of genes, protein synthesis, viruses, immune system and cancer, mathematical theories of carcinogenesis, radiology and cancer.

In Chapter II, mathematical models of cell growth are discussed. Since the length of the cell cycle is not constant, Section 2 tries to reconcile the plethora of deterministic and stochastic approaches to discrete and continuous cell models existing in the literature. Sections 3 and 4 contain the derivations of common growth laws and their use to systematically represent data for experimental tumor systems. However, the clinical applications of mathematical growth models require the development of new methods of determining (solid-) tumor size.

Chapter III summarizes several models used in cell cycle analyses, radioactive-

labelling experiments and chemotherapy. A number of experimental results are cited which were processed by solving the equations presented.

In the next chapter, mathematical formulas used in quantitative studies of cell kinetics based on autoradiography are derived. Mathematical and biological problems inherent in labelled mitosis analyses are critically evaluated to stimulate further research.

Chapter V deals with methods for quantitating analyses of synchronous cell populations. A rapid method for measuring the degree of synchrony with respect to different stages of the cell cycle is presented in Chapter VI (Flow Microfluorometry).

Chapter VI contains information about selected topics of control theory. In a separate Appendix (by W. Düchting) there is an outline of applications of control theory to normal and malignant cell growth.

Chapter VII (Towards Mathematical Chemotherapy) shows how optimal control theory has been and can be applied to obtain optimal drug schedules. The cornerstone of optimal scheduling is that tumor cells usually exhibit different growth kinetics than critical normal tissue. The preceding chapters are essential to understand the sections of the part entitled Growth Laws and Cycle Nonspecific Cancer Chemotherapy, Cycle Specific Chemotherapy, Pharmacokinetics.

The last chapter (Mathematical Models of Leukopoiesis and Leukemia) critically reviews steady-state models of the hemopoietic system; kinetic model of neutrophil production; a chemotherapy model of acute myeloblastic leukemia; models of chronic granulocytic leukemia and acute lymphoblastic leukemia; a comprehensive computer model of granulopoiesis and cancer chemotherapy. Many of the computer models have only been used for theoretical studies. Moreover, there is no indication of how to apply these models to quantitate the effects of a particular drug. These difficulties are discussed and possible avenues of research suggested.

The book combines the advantages of a comprehensive review and a practical guide. Thus, it can be recommended to applied mathematicians as well as to intelligent cell biologists or chemotherapists.

F. BARTHA

Vitamin D. Metabolism and function. Monographs on Endocrinology series Vol. 13, by H. F. DeLuca. Springer-Verlag, Berlin–Heidelberg–New York 1979, 80 pages, with 14 Figures

This slender volume presents a concise yet very informative summary of the discovery and current knowledge of what is now termed as the "Vitamin D endocrine system" — one of the most puzzling discoveries in the past decade, because it revealed striking similarities of 1,25-dihydrocy Vitamin D_3 (1,25-$(OH)_2D_3$) to steroid hormones both with respect to mode of biosynthesis and mechanism of action.

First the author discusses in a logical and comprehensive way the historical aspects and the chemical structures of compounds with Vitamin D activity. Next, the formation and storage of Vitamin D_3 are described and the cardinal role played by the liver and the kidney in the production of various hydroxylated Vitamin D_3 derivatives is highlighted. Perhaps the most intriguing chapter is the one that reports on the regulation of Vitamin D metabolism and the specific roles of parathromone, calcitonin, phosphate and sex hormones in calcium homeostasis. This chapter is followed by a treatise of the present trends of research on the mechanism of action of Vitamin D. Here ample space is devoted to the characteristics of a 1,25-$(OH)_2D_3$ receptor and the induction of a specific Ca^{2+} carrier mechanism in the intestinal villus cell.

At this point the reader had gained a detailed enough insight into the interplay of various hormones in the maintenance of calcium and phosphorus balance to be able to predict possible malfunctions in this complex regulatory system and some possible ways to correct them. Indeed, the last chapters focus on these problems, namely, on the practically useful analogs of 1,25-$(OH)_2D_3$ (the most significant of which is 1-OH-D_3) and the practical benefits of Vitamin D compounds, especially 1,25-$(OH)_2D_3$, in diseases such as hypoparathyroidism, renal osteodystrophy, Vitamin D — dependency rickets and postmenopausal osteoporosis.

The text is supplemented with useful illustrations and an extensive bibliography

that makes the book a valuable source of references for the investigator.

The author makes it clear that although the number of unsettled questions is apparently high, there are no foreseeable dead ends in most of the many ways in which Vitamin D is studied and the perspectives of new insights into the cause and treatment of several bone diseases are really exciting. The author himself emphasizes that his monograph is in a sense a progress report of a study of the metabolism and function of Vitamin D and its implications in medicine. Evidently, the findings brought together in this book form a basis for a better definition of the calcium and phosphorus homeostatic mechanisms. The results may lead in the near future to more sophosticated information giving not only a firm scientific support to the treatment of metabolic bone diseases but also capable of rendering it more effective.

This book should be a mandatory reading for those biochemists, chemists, biologists and physicians whose everyday work requires a novel way of thinking of Vitamin D. I also suggest it to lecturers of medical biochemistry and particularly to those who may want to gain access to a scope of investigations that will be feasible in this field in the near future.

M. TÓTH

Mechanism of Action of Antibacterial Agents. Vol. V/1, 2, and *Mechanism of Action of Antieukaryotic and Antiviral Compounds. Antibiotics.* by F. E. Hahn (editor), 270 pages, 80 figures and 470 pages, 122 Figures, 53 Tables respectively. Springer-Verlag, Berlin—Heidelberg—New York 1979.

In 1967 when the first volume of the series "Antibiotics" was published the editors claimed to restrict the scope of all volumes to covering only antibiotics in the classical sense, defined as metabolites produced by certain microorganisms to inhibit the metabolism of another one or others. In 1979 Dr. Hahn, editor of the fifth volume, already could not accept this terminology and arranged chemotherapeutics principally according to their target (bacterium, virus) besides their mechanism of action. In the individual volumes the compounds discussed are listed in alphabetical order.

It is a rewarding task to compile a comprehensive work on such a rapidly developing, popular subject like antibacterial and antiviral agents, but very unrewarding at the same time, since in a couple of years it requires total revision or, at least, thorough enlargement. Thus, for example, the first volume edited in 1967 concentrated on the problems of isolation, structure and mode of action, while in recent years interest has ever increasingly been focussed on the molecular aspects of antibiotics action. Thus, it has become necessary to supplement our previous knowledge even in the case of such well known antibiotics as tetracyclines and streptomycin. The editor, in turn, omitted penicillin and chephaloporins from the volume arguing that a number of comprehensive fundamental works have been published on these lactam-type compounds. Also, some other pharmacologically important synthetic compounds, like nitrofuranes, ethambutol, 8-azaguanine have been included because their way of action is similar to that of antibiotics.

This is the first book that illuminates structure and mode of action of some toxins among the substances affecting eukaryotes. As the author did not intend to make an encyclopedia of poorly defined substances with unknown mode of action, some substances, such as interferon, have been left out from the lots of antiviral substances.

The 20 and 22 comprehensive chapters of the two volumes, respectively, are the works of numerous noted biologists, pharmacologists, physicians and biochemists. Thus, one of them regards more important to discuss the molecular mechanisms, the other prefers the clinical aspects, a third the change of a certain effect following chemical modification. It is worth mentioning the short and clearly written chapter on bacitracin by D. R. Storm and W. S. Toscano, Jr. The authors summarize the main problems of isolation and structure of bacitracin, discuss its biosynthesis on the basis of experimental data up to 1974—75 and analyze its mode of action from several aspects (effect on membrane permeability, on peptidoglycane biosynthesis etc.).

E. P. Bakker compiles into numerous Tables the most important ionophore antibiotics together with their ion selectivity. He gives a detailed account of the mechanism of action of the particular antibiotics stressing the fact that, although their therapeutic or pharmacological usefulness is still limited, ionophore antibiotics have become important tools in cell research.

The mechanism of action of polyene antibiotics, as supported by spectroscopic and calorimetric data, has been reviewed by R. W. Holz. With regard to their mode of action these compounds are closely related to ionophoric antibiotics. However, as their target may be eucaryotic organisms the polyene antibiotics (nystatin, amphotericin B and filipin) are the subject of Volume 2.

J. J. Roberts helps the reader get an insight into the world of platinium complexes that readily react with DNA and, on the basis of their mode of action, can be related to the large family of antibiotics affecting protein synthesis.

This chapter is followed by an up-to-date description of toxins from the viewpoint of protein chemistry, with due emphasis on their mode of action. The reader hardly recovers from his amazement of how many subjects can be brought together in a book on antibiotics, he soon comes across a review on the alkaloid chinin and, another one on vinca alkaloids. He has the feeling of having lost track of classical antibiotics. But then, he reads again chapters on the "classical" antibiotics showdomycin, phleomycin, neomycin and related compounds with an account on molecular mechanisms.

„Antibiotics V/1–2" is a very comprehensive treatise which is worth reading even out of curiosity, but also experts of a given field can use it since the detailed bibliography collected up to 1978 is of great help.

Katalin MAROSSY

Molekular und Zellbiologie (Molecular and Cell Biology) by Von Sengbusch, P., 671 pages, 616 Figures. Springer-Verlag Berlin–Heidelberg–New York 1979.

The word "biology" nowadays has almost exclusively been used as a collective noun to denote a number of scientific areas dealing with living organisms. The reason of this is that the separate branches and fields have developed into individual entities. Thus, it is a formidable effort to select the most important results from the almost infinite mass of data from diverse research fields in such a way that they should form an integrated picture for experts, or students going to be scientist.

As demonstrated by this volume P. von Sangbusch has succesfully overcome this difficulty in the field of molecular and cellular biology. A key to his succes may be that, violating tradition, he restricts himself, in most cases to the discussion of only up-to-date results.

He does not deal with the origins or the first discoveries of a given branch of science nor with its subsequent development. So he is able to omit many superfluous data and a description of out-of-date conceptions. This, in turn, assures for the author all the advantages of conciseness and modernness, an invaluable ease for the undergraduate student. It is clear that if one wishes to get deeper into the details of a given field of science one cannot miss learning of the intellectual, sometimes highly controversial, history of that particular branch. This, however, must be taken from other sources.

A further merit of the volume that must be emphasized is the rich array of illuminating, excellent illustrations. The great number of drawings, sketches and diagrams are supplemented with a number of light and electron microscopic pictures helping the reader allot the relationships inside the cell to their proper place so to say visually.

The subject is divided into seven major chapters.

First, the properties and functions of nucleic acids are described indicating the realization of informations with regard to the origin of the characteristic properties of living matter residues in these molecules. Next, in Chapter 2, the characteristics of proteins, the most important group of molecules with cellular functions are described. Here, a broader scope from the viewpont of function may have served better.

A relatively lengthy chapter deals with the structure of membranes with respect to the functioning of pumps and to the importance of membrane receptors.

Moving from the simple towards the complex, next the cytosceletal and contractile structures are discussed in a separate chapter. Even if such an accentuation may not be fully justified, the intensive research work and the great bulk of new results in this field can explain this preferential emphasis.

The last three Chapters analyze complex biological systems. It is worth considering that, among the supramolecular structures, the author reviews only those which are somehow correlated with the genetic information, namely, the ribosomes, the chromatin and the cell nucleus, the viruses etc. What is even more unusual, mitochondria are discussed in detail only in terms of their protein synthetizing ability. Consequently here, but also in the aforementioned case, questions such as ways of meeting the energy requirement of cells for the proper functioning of the living organism and other problems, remain in the background.

Chapter 6 describes the structure and function of cells with regard to their development, regulation and other phenomena, while the last Chapter informs us about the multicellular systems.

It is evident that to reach a certain goal other claims must be neglected. P. von Sengbusch solved this problem by making a compromise in such a way that the reader's loss is minimal. Modern in style with excellent and highquality illustrations and didactically also very organized, this book provides its user with the up-to-date results of biology.

P. ELŐDI

Comparative Physiology and Evolution of Vision in Invertebrates. A: Invertebrate Photoreceptors. Handbook of Sensory Physiology, Vol. VII/6A, by H. Autrum (ed.). Springer-Verlag, Berlin–Heidelberg–New York 1979

The volume is introduced by Prof. H. Autrum by reviewing in Chapter 1 the comparative physiology and evolution of vertebrate and invertebrate photoreception. Variability in ecological adaptation, structural types of photoreceptor cells and primary photoreceptive processes are compared and summarized.

In Chapter 2, photic responses and sensory transduction in motile protists are discussed by B. Diehn. The chapter begins with a description of the terminology, experimental methods used for studying photomovements as well as cellular organelles and receptor pigments involved in sensory transduction. The author then examines the problem whether there is a link between systems mediating photosynthesis and photomovement. Behavioural mechanisms and interactions of photosensory and other (mechanical, chemical, gravitational) stimuli are discussed next. The chapter ends with a short reviewing outlook of experimental approaches and open problems.

Chapter 3 discusses the effects of optical filtering on eye function (W. H. Miller). The functional significance of such filtering processes is that information that is passed from the front of the cornea to the photoreceptor structures is the only sensory information available to the visual system. This chapter deals with the physical basis of transparency of ocular media, with interference and absorption filters (pupillary function of screening pigments, yellow absorption filters, scattering, reflection suppression and enhancement, tapeta), with the filtering effect of the spatial arrangement of photoreceptors with regard to the maximum amount of spatial information passed. Waveguide effects (determination of directionality, waveguide spectral effects, optically separate channels) are treated at the end of the chapter. In addition to describing the particular phenomena each section tries to explain their functional significance and mechanism.

A separate chapter (Chapter 4 by K. Hamdorf) is devoted to the physiology of invertebrate visual pigments. Analogies and deviations from the vertebrate pigments in basic light and dark reactions are described on a few selected examples of cephalopods, crustaceans and insects. The author discusses electrophysiologic findings (early receptor potential, prolonged depolarization afterpotential and late receptor response) that yield information on visual pigment function. Diverse and sometimes contradictory data obtained with various species are united in a model to explain the photopigment system universally. Separate sections discuss the multichromatic visual systems in flies and Deilephila as well as the evidence

for retinaldehyde as the chromophore in invertebrate visual pigments. The microvillus of the invertebrate photoreceptor is considered as the functional and structural unit for which a model is proposed by analogy with the discs of vertebrate photoreceptors. A voluminous part of the chapter is devoted to the mathematical treatment of and calculations on equilibrium of interconvertible visual pigments as well as on spectra and quantum efficiency.

Chapter 5 (by A. W. Snyder) offers a personalized concept of the physics of vision in compound eyes. In the section about visual acuity, factors limiting the resolving power are considered and used to explain the design of a compound eye. Such limitations are: finite angular spacing of ommatidia, diffraction and finite rhabdom diameter, finite integration time, photon noise to contrast sensitivity. In the next section, various strategies for design are examined that allow an animal to have sufficient resolution over a range of contrast and intensity. We also learn how neural processing compensates for optical blur and photoreceptor noise. The section about sensitivity provides a theoretical basis of evaluating the absolute sensitivity of the ommatidium. In this section, the light-gathering capacity, morphological characteristics and dichroism of the rhabdom as well as the chromophore alignment are considered. The section about the optical properties of the photoreceptor membrane reviews dichroism and birefringence and compares vertebrate and invertebrate photoreceptors. The last section deals with the wave guide property of photoreceptors and with its influence on the optical characteristic of the rhabdom.

Chapter 6, written by M. Järvilehto, deals with electrophysiological findings on invertebrate photoreceptors. It is a brief and clear presentation of the subject with emphasis on correlation with anatomical data. The chapter begins with a short survey of photoreceptor organization, followed by a description of the method of intracellular recording with microelectrodes. The electric activity of photoreceptor cells is clearly discussed, generation of electric responses, early receptor potential, quantum bumps, receptor potential and spikes are described. Because of univariance of light perception, one cell is not capable of finding out all informations contained in light radiation. Therefore cooperation of several cells is needed the form of functional units. Emphasis is laid therefore on the functional organization and interaction of photoreceptor cells. Anatomical contacts, optical and electric coupling, electric field interaction and synaptic contacts are discussed in this context.

An interesting chapter (Chapter 7 by D. G. Stavenga) is devoted to the pseudopupil of compound eyes, an optical phenomenon that provides direct insight into the structure and function of the living eye. After an introductory treatise on optics and mosaic organization of compound eyes, the fundamentals and various varieties of pseudopupils are discussed. The author describes how pseudopupil phenomena can be used in compound eye research: (i) in analysis of the spatial organization of the retinal samplers and of the optical properties of retinular cells, (ii) in studies on adaptation processes and (iii) in the sophisticated techniques of selective stimulation of individual receptors.

There is a remarkably clear presentation on apposition and superposition eyes (Chapter 8 by P. Kunze). The chapter begins with a tribute to S. Exner's almost 90-year-old observations about the optics of compound eyes. Several examples of superposition eyes are reviewed by describing anatomy, optical parameters of refractive media, ray path tracing and experimental tests (eye chamber preparation, eye glow, optomotor experiment). A similar line is followed in the discussion of apposition eyes, with special emphasis on eyes with open rhabdoms (single receptor stimulation) and of the neural superposition type.

We find an excellent review (Chapter 9 by R. Menzel) about spectral sensitivity and colour vision of invertebrates. There is a detailed phylogenetic comparison of various invertebrate species (lower invertebrates, Arthropoda, Mollusca, Echinodermata, Crustacea, Chelicerata and Insecta). Among these, insect photoreceptors are especially broadly discussed: possible mechanisms underlying the broadening of spectral sensitivity and the appearance of secondary peaks, self-screening of photopigments, positive electric coupling between photoreceptor

cells, more than one pigment in one visual cell, screening pigments as well as mechanisms causing narrower spectral sensitivity than rhodopsin resonance functions, spectral transmission of the dioptric apparatus, reflection of the tapetum, selective filtering by screening pigments and negative electric coupling are enumerated. Five additional chapters are concerned with the spectral sensitivity of selected insect species, distribution of colour receptors, spectral phototaxis, spectral properties of insect ocelli and of extraretinal photoreception. There are interesting chapters about central processing of colour information and wave-length specific behaviour. The idea that chromaticity-coding visual systems have evolved because they specifically elicit appropriate behaviour patterns (UV-VIS antagonism) is an attractive one.

In Chapter 10 (M. Yoshida), structural and functional aspects of various types of extraocular photosensitivities are considered. They are divided into direct responses and diffise, neural and neuronal sensitivities. In dermal photoreception, two types are distinguished: reflex responses and coordinated movements for which a central mechanism is needed. Photosensitivities in the nervous system can be considered as neural and neuronal sensitivities with three good examples of identified photoexcitable neurons for the latter. There are also photoreceptor cells which have elaborated cellular differentiation for photoreception but are scattered in various parts of the organism either as single cells or organized as primitive ocelli. The dermal and ocular photosensitive systems are compared from a functional point of view.

This handbook is concluded by two short chapters, the first of which discusses the relation between extraocular light receptors and circadian rythms (Chapter 11 by M. F. Bennett) in various invertebrate classes. In the other chapter (Chapter 12 by M. Heisenberg) a genetic approach to the visual system in presented. Three subjects are considered. The first one concerns mutants with defects in the retinula cells and their role in the analysis of the transducer process, the second one deals with mutations that specifically eliminate certain receptor types and the last one describes dissection of behaviour.

P. RÖHLICH

Normale und Pathologische Physiologie der Haut, II. *Handbuch der Haut Geschlechtskrankheiten, Ergänzungswerk*, 1/4 A:*(Handbook of Skin and Veneral Diseases, Suppl.* 1/4A: *Normal and Pathological Physiology of the Skin*, II.*)* by J. Jadassohn, Springer-Verlag, Berlin—Heidelberg—New York 1979.

The editors of this book of 606 pages are E. Schwarz, H. W. Spier and G. Stüttgen. It appeared 15 years later than the previous volume (1/3) and has been compiled mostly according to the intentions of late H. W. Spier who died in 1975.

The contributors to the individual chapters are E. Schwarz, R. Bauer, F. Klaschka, M. Gloor and J. Horacek, H. Schliak and R. Schiffer as well as E. G. Jung and E. Bohnert. The book has a similar lay-out as the previous volume; a new chapter has been added on the regulation of epithelial cell cycle.

The chemical–physiological aspects of structure and metabolic processes are dominant in each topic.

In the Chapter "The biochemistry of keratinization and hyperkeratinization of the epithel" the biochemical interest of the author (E. Schwarz) is especially apparent. A lucky approach, because on a purely morphological basis without biochemistry, this problem cannot be understood.

In all instances the author discusses both the normal and pathological functions, the postsynthetic changes of proteins, as well as their phylogenesis. The clear and concize text documented by all the essential references contains 39 pictures and 30 tables helping the reader to a fuller understanding of the subject.

"*The neurophysiology and pathophysiology of perspiration*"

Part C deals with the functions of eccerine glands. Disturbances in sweat secretion observed in specific neurological diseases are described in detail. The sweat secretion of the palm and the sole takes place both spontaneously and on emotional triggers and is not subjected to thermoregulation. The chapter contains 81 photographic pictures and 2 tables.

In the chapter "*Principles of the physiology of Stratum Corneum*" the author gives a detailed account of the process of kreatini-

zation. Data on the barrier function of the ephithel are summarized in an emply illustrated section. This chapter, in addition to a number of up-to-date experimental procedures, contains also the description of a method for measuring the transparency and thickness of the epithel. Twenty one pictures and 9 tables supplement this chapter.

In the chapter "*The lipid coat of the skin*" the protecting function of the skin is discussed in great detail.

The author describes the composition of lipids, its changes during the process of ageing and shows that there is no significant difference in the lipid composition of the skin between young or old people.

There is no unanimous opinion with regard to the protective role of the lipid coat of the skin. On local medical treatment care must be taken of the existing state of the lipid coat and its changes during therapy. The chapter is completed by 23 pictures and 23 tables.

The chapter entitled "*The light and the skin*" describes DNA damages that occur on ultraviolet irradiation and some recent results of DNA repair studies. The author emphasized the importance of the changes in the dermis and epidermis during light-induced dermatoses. An extensive list of references helps the specialist learn about the details. This part includes 27 pictures and 13 tables.

This manual has piled up a mass of information useful not only to researchers but also to practising clinicians.

E. NAGY

Blut and Blutkrankheiten (Blood and Blood diseases) Leukämien, in Schwiegk (Publisher) *Handbuch der inneren Medizin (Handbook of Inner Medicine)* Vol. II., Part 6. by Begemann, H. (ed.), Springer Vlg. Berlin—Heidelberg—New York 1978, 635 pages, 77 Figures (part of them in color) and 31 Tables.

Written by 9 authors, the nine chapters of the book summarize our present knowledge of leukaemia. The reader is informed about the history of research concerning leukaemia, the determination of the syndromes and about the epidemiology of the disease (Chapter 1), as well as about the etiology of leukaemia (Chapter 2), complications and therapy (Chapter 4), acute cases (Chapter 5), the proleukaemic state (Chapter 6), the di Guglielmo syndrome (Chapter 7), the chronic myeloid (Chapter 8) and lymphoid cases (Chapter 9).

The Chapter on the biochemistry of leukaemic cell, containing 58 pages and making a mere 10 per cent of the volume page number, was written by J. B. Obrecht. Obviously, in a journal of biochemical interest this part needs a more detailed comment. The ratio of this chapter to the others indicates that our knowledge concerning the principles of the biochemistry of the leukaemic cells is relatively limited. Even the available data are considerably scattered and too much mosaic-like to use them for sketching even the most essential contours. As an excuse for the investigators may serve the fact that "leukaemia", similarly to the word "cancer" is a collective noun implying a great number of processes all differing in etiology and pathology. On the other hand, it should also be noted that, with the spreading of environmental pollution (radioactive and X-ray radiations, several chemicals etc.) an increase in risk factors and, as a consequence, a growth of the number of leukaemic patients may be expected. This justifies the claim that researchers in the field of medical biochemistry should intensify their efforts in exploring this subject, as well as in studying the other tumor diseases. It is a merit of the Chapter that, besides its conciseness, it promotes a primary orientation of the reader by an ample number of references.

Besides the difficulty of having to present a unifying picture from the sporadic information available, in spite of the large number of bibliographical data at his disposal, the author, J. B. Olbrecht has been faced with another obstacle. Namely, that his would be readers, probably mostly medical doctors, are involved in everyday clinical practice and must have forgotten almost every bit of their basic knowledge of biochemistry. That is why the author has been compelled, in the frame of the otherwise limited space, to reiterate some information that is common-

place to the expert and to deal with matters of textbooks.

Most data in the literature are descriptive and many books deal with differences in composition between normal and pathological leukocytes. Obviously, it is of primary importance to collect and to learn these data, yet, whether an observed change in the amount of a given compound can be attributed to primary or secondary causes, cannot be judged.

One-third of the Chapter conceivably, deals with nucleic acids. Evidently, multiplication of leukaemic cells rests on the synthesis of purine and pyrimidine bases, and that of polynucleotides. That is why in almost all leukaemias these mechanisms exhibit an overincreased activity as compared to those in normal cells. The author attributes great importance to the presence of reverse transcriptase (revertase) and to the absence of 5' nucleotidase in the membranes of leukaemic cells.

The metabolism of proteins (amino acids) has been treated relatively briefly as compared to that of nucleotides save, for known reasons, a more detailed discussion of data concerning the metabolism of asparagine. Vitamins, especially vitamin B_{12}, have been reviewed in a section of similar extent as the proteins.

The final conclusion of the author, namely, that in spite of the recent, highly accelerated progress in this field there are many gaps in our knowledge, can only be approved.

P. ELŐDI

Mathematical Aspects of Reacting and Diffusing Systems, by Paul C. Fife, in Lecture Notes in Biomathematics Vol. 28 Managing Editor: S. Levin, Springer-Verlag, Berlin–Heidelberg–New York 1979 (165 pages).

There are certain analogies between chemical reactors and living systems. Some of these analogies are rather obvious: a living organism is a dynamic structure built of molecules and ions, many of which react and diffuse. Others are less obvious; e.g. the electrical potential of a membrane can diffuse like a chemical and can interact with different chemical species which are transported through the membrane. Such phenomena like these play an important role in the propagation of nerve signals. Some other analogies can be found on higher levels of organization. These levels involve communities and populations.

Analyzing and modelling the dynamics of chemical mixtures by means of differential equations are the main concern of chemical engineering theoreticians. The equations often take the form of systems of parabolic partial differential equations or reaction-diffusion equations. For all of the above cases the simplest continuous space-time interaction-migration models have the same general appearance as those for the diffusing and reacting chemical systems. These systems are the objects of Fife's excellent book.

The notes on which this text is based were originally written by P. C. Fife for a course on reaction-diffusion systems at the University of Arizona in spring, 1977. These notes have been extended and references up to 1979 are included in the volume.

Chapters of the book: 1. Modelling Considerations, 2. Fisher's Nonlinear Diffusion Equation and Selection-Migration Models, 3. Formulation of Mathematical Problems, 4. The Scalar Case, 5. Systems: Comparison Techniques, 6. Systems: Linear Stability Techniques, 7. Systems: Bifurcation Techniques, 8. Systems: Singular Perturbation and Scaling Techniques, 9. References to Other Topics. In the first two of the nine chapters Fife shows how reaction-diffusion systems arise as models. The mathematical methods for handling the equations are dealt with in Chapters 3—8. These chapters can be followed even with a moderate mathematical knowledge. Chapter 9 gives insight into some closely related problems works, and areas not covered by the book.

The logic of the book is clearly stated. Gaps in the field are pointed out. References are up-to-date and carefully selected. The text is readable. Altogether a buy worth the money. Moreover, the book should perhaps be used as a teaching aid in chemistry, biochemistry, biophysics, populations genetics just to keep up with recent knowledge and development in the field.

J. HAJDU

Lymphocyte Hybridomas by F. Melchers, M. Potter, N. L. Warner (eds). Springer-Verlag, Berlin–Heidelberg–New York 1978, 246 pages with 85 figures.

The book contains 38 reports of the second workshop on functional properties of tumors of T and B lymphocytes, held in Bethesda in April, 1978. Most of the studies deal with the fusion of normal and malignant lymphocytes and with the resulting hybrid cells producing monoclonal antibodies. The malignant parent lines included mouse mastocytomas and lymphomas while mouse spleen cells or purified B and T lymphocytes were the normal parent lines used for cell hybridization. Very few contributors used human cell lines for cell fusion. Each report provides sufficient technical detail and references concerning the techniques of cell fusion, selection and characterization of hybrid cells. The major technical problem in this field is the difficulty to maintain a functionally stable hybridoma. It has been suggested that recloning early in the propagation of the specific clone is advantageous for maintaining a stable clone. The fusion of normal and malignant lymphocytes opens the possibility of obtaining monoclonal antibodies and other specific products of lymphocytes to any known or unknown antigenic specificity including many that are of practical relevance to both experimental and clinical immunology, molecular biology and cell biology. The book is valuable for immunologists, geneticists and scientists working in cancer research.

O. Csuka

Biological Functions of Proteinases by H. Holzer and H. Tschesche (eds), Springer-Verlag, Berlin–Heidelberg–New York 1979, 284 pages with 142 Figures

The volume contains 26 papers on proteinases, presented at the 30th Mosbach Colloquium in April 1979. The various contributions touch upon many areas of protease research, the major emphasis, however, is on the role of intracellular proteases in general protein turnover, in controlling the steady-state concentration of proteins in mammals and microorganisms. Several papers deal with control mechanisms that permit selective elimination of abnormal proteins or regulate the intracellular degradation of proteins during starvation and differentiation of cells. The role of endogenous inhibitors in regulating protein turnover during normal and pathological conditions is also discussed. Several contributions cover proteases that carry out highly specialized regulatory functions by limited proteolysis of specific target proteins. The regulatory function of limited proteolysis in transporting proteins across membranes during secretion or entry of proteins from cytoplasm into mitochondria, the function of proteases in protein processing and in assembly of viruses are discussed in addition to the better known significance of limited proteolysis in the regulation of the complement reaction, control of blood pressure, blood coagulation, fibrinolysis etc. This book, by providing an overview of our present knowledge of the varied regulatory functions of proteases, helps delineate the future trends in protease research and is thus useful for those interested in the function of proteases in biological control mechanisms.

L. Patthy

Reviews of Physiology, Biochemistry and Pharmacology 81. (Formerly Ergebnisse der Physiologie, biologischen Chemie und experimentellen Pharmacologie.) by R. H. Adrian, E. Helmreich, H. Holzer, R. Jung, O. Krayer, R. J. Linden, F. Lynen, P. A. Meischer, J. Piiper, H. Rasmussen, A. E. Renold, U. Trendelenburg, K. Ullrich, W. Vogt and A. Weber (eds.). Springer-Verlag, Berlin–Heidelberg–New York 1978

Langman, R. E. is the author of the first part entitled "*Cell-Mediated Immunity and the Major Histocompatibility Complex*". The aim of his review is outlined in the prologue. Data about the cluster of genes in the region of the species major histocompatibility gen complex are dealt with as the main area having provided information on the principles of control and regulation of the immune system. The histocompatibility-2 (H-2) complex is regarded as the basic starting point in Langman's review. In the

"Background and Current Concepts" he writes a synopsis of H-2, H-2 restriction as well as some earlier models, namely the intimacy model, the modified-altered self-model and the complexed-altered self-model. In the second chapter the dual receptor principle is discussed in detail. This chapter is completed by a schematic representation of dual-recognition model. The third chapter deals with the "Evolution of Cell-Mediated Immune System". In this chapter the use of the decimal system is especially rewarding because it makes the diverging topics more discernible. This chapter is followed by a fine discussion. The epilog gives a nice overview of the topic, emphasizes the significant outcomes. The chapter is completed by a reference list.

The second part of the book is about the "*Central α-Adrenergic System as Targets for Hypotensive Drugs*". The author of this part is W. Kobinger. In his study he gives an overview of the two well known antihypertensive drugs, clonidine and α-methyldopa. The main chapters are: "Clonidine-like Drugs", "α-Methyldopa", "Central α-Adrenoreceptors as Mediators of Cardiovascular Depression" and "Possible Role of Noradrenaline as a Central Neurotransmitter in Cardiovascular Regulation". After the introduction the chemical structure of several clonidine-like drugs is given, including the two possible tautomeric forms of clonidine as well as its aplanar steric conformation in the protonated form. The only thing which seems to be missing from the tables and figures is a supply of some particular characteristics, such as solubility and other physicochemical data. The second chapter gives an excellent summary of peripheral effect, cardiovascular depression, hypotensive and bradycardic effect of clonidine as well as the mechanism of cardiovascular transmission. This interesting chapter is concluded by the subchapter: "Hypotension in Human." The third chapter is concerned with the hypotheses relating to the mode of action of α-methyldopa, its peripheral effect and central site of action. The fourth chapter is the most well arranged part of this excellent review and it gives an easily understandable discussion of the topic. This chapter — like the other — cites a large number of the author's own papers. The part concludes with comparative analysis of the role of noradrenaline and a reference list.

One short part of the book is entitled: "*Structure and Function of Nuclear Ribonucleoprotein Complexes*" written by P. C. Henrich, V. Gross, W. Northemann and M. Scheurlen. The method of isolation and properties of nRNA particles are summarized in tables, the structural and functional aspects of nuclear ribonucleoprotein particles are discussed in detail and the text is nicely documented by figures. The effect of hormones and drugs on nRNA particles is also discussed. The "conclusion" chapter does not only complete this chapter but also gives initiatives for further investigations.

The fourth paper of the book is about the "*Action and Uptake of Neurotransmitters in CNS Tissue Culture*" written by L. Hösli and E. Hösli. The work starts with an overview of papers on nervous tissues cultured in vitro, the cytologic and hystochemical properties of neurons and glial cells in tissue culture. Most microscopic pictures presented here are from different papers of the two authors from 1973, 1974 and 1975. They are very good quality reproductions. The uptake of amino acid transmitters (GABA, glycine, glutamic acid, aspartic acid) as well as that of noradrenaline, 5-hydroxytriptamine and dopamine were detected by autoradiography using tritium labelled forms of these compounds. Light microscopic autoradiographs illustrate the detailed findings. The electrophysiological properties of cultured neurons and cultured glial cells are also discussed. The chapters dealing with the two latter topics are especially packed with references which supply an enormous amount of information but lack perspicuity in want of arrangement in tabular form. The effect of inhibitory amino acids, excitatory amino acids as well as the effect of acetylcholine and monoamines on cultured neurons are explained in detail. The interesting final chapter states that although the amino acid transmitters are taken up by cultured glial cells, electrophysiological studies have shown that they do not act on the membrane potential of glial cells. At the same time, acetylcholine was found to have depolarizing and hyperpolarizing effect on glial cells. The summary and conclusions are followed by a reference list with almost 320 cited works

that completes the part about the uptake and action of neurotransmitters in CNS tissue cultures.

All four parts of the book give reviews about very important and frequently mentioned fields of science. The individual parts deal with very distinct topics and yet have been written practically in the same style. The introduction give a basic overview of results of general interest and are followed by a detailed description of the respective data and theoretical considerations supported by both with schematic figures and photographic pictures. The huge amount of knowledge is sometimes too concentrated for optimal understandability but is generally arranged in table. The literature references are complete and facilitate any further and more extensive study.

H. KALÁSZ

Bacterial Metabolism by G. Gottschalk. Springer-Verlag, New York–Heidelberg–Berlin 1979. pp. XI + 281

This book has been written for students who are taking a course in bacterial metabolism. It is divided into ten chapters. Every chapter concludes with a summary.

Chapter 1 gives a general introduction to the nutrition of bacteria. First the major and minor bio-elements are described. The relevant Tables point out their functions and metabolism. Then the two basic mechanisms of ATP synthesis (electron transport phosphorylation and substrate level phosphorylation) are briefly summarized, followed by an overview of nutrients as energy sources. A glimpse on growth factor requirements of bacteria completes the material concerned with bacterial nutrition.

After this chapter there comes a bifurcation of the logical sequence of the chapters, based on the fact that, if glucose is the substrate, about 50% is oxidized to CO_2 resulting in enough ATP (Chapter 2) to convert the other 50% into cell material (Chapter 3).

Chapter 2 examines how *Escherichia coli* synthesizes ATP during aerobic growth on glucose. The reactions involved in this process are divided into and dealt with according to the following blocks of reactions: A. Transport of glucose into the cell by the phospho-enolpyruvate phosphotransferase system. B. Degradation of glucose-6-phosphate to pyruvate via the Embden–Meyerhof–Parnas pathway. C. Oxidative decarboxylation of pyruvate to acetyl-coenzyme A by the pyruvate dehydrogenase complex. D. Oxidation of the acetyl moiety of acetyl-coenzyme A to CO_2 via the tricarboxylic acid cycle and E. Oxidation of the reduced coenzymes formed in steps B to D in the respiratory chain (formation of ATP). Step E is treated in most detail by the author.

Chapter 3 reviews the biosynthesis of *E. coli* cells from glucose. A very lucid general scheme (Fig. 3.1) helps the student grasp the ways of biosynthesis of cell material from glucose. This chapter is very condensed and diversified at the same time. Practically it covers most aspects of what is generally termed intermediary metabolism and also draws a sketch of molecular biology. A mere enumeration of the topics included will perhaps give a hint about the reason of the reviewer's dissent concerning the didacticism of this particular chapter: composition of *E. coli* cells, assimilation of ammonia, assimilatory reduction of sulfate, biosynthesis of amino acids, formation of pentose phosphates and $NADH_2$ synthesis of ribonucleotides and deoxyribonucleotides, biosynthesis of lipids, formation of carbohydrates, and synthesis of polymers (lipids, periodic macromolecules, informational macromolecules).

The above three chapters can be considered as a general introduction to bacterial metabolism. A transition to subsequent chapters which are, except Chapter 7, of a more specific nature with respect to metabolic particularities of bacteria, is represented by Chapter 4. This gives a very brief summary of the aerobic growth of *E. coli* on substrates other than glucose (fructose, lactose, acetate, pyruvate, and malate).

Discussion of the topics that are indeed specific to bacteria is introduced by two chapters on aerobic heterotrophs.

The first of these, Chapter 5, is about the metabolic diversity of aerobic heterotrophs. Here the author has to repeat, with a more extensive scope in mind, almost all topics he had written about in the earlier chapters which referred only to *E. coli*. He summarizes our present knowledge of the different mecha-

nisms for the uptake of substrates (passive diffusion, facilitated diffusion, active transport and group translocation), the Entner—Doudoroff pathway of carbohydrate breakdown, the pentose phosphate cycle, and the methyl glyoxal bypass. Then he deals with the diversity of energy metabolism (pyruvate dehydrogenase, tricarboxylic acid cycle, respiratory chain), the dissimilatory reduction of nitrate, alternate anaplerotic (replenishing) sequences (phospho-enol pyruvate carboxylase, pyruvate carboxylase), and biosynthesis of monomers and polymers.

The second chapter on aerobic heterotrophs, Chapter 6, is about their catabolic activities. After a bird's-eye view on the degradation of polymers by exoenzymes, metabolic pathways related to growth with amino acids, organic acids, aliphatic hydrocarbons, aromatic compounds, and C_1—compounds, respectively, are reviewed. A separate part is devoted to incomplete oxidations.

In Chapter 7 the reader is compelled to turn back his mind to some basic metabolic concepts, this time about regulation of bacterial metabolism. In the frame of regulation of enzyme *synthesis* by induction and repression, the phenomena of enzyme induction, catabolite repression, end product repression as well as synthesis of enzymes of central pathways are dealt with. The other regulatory process, regulation of enzyme *activity*, is discussed under the headings of feedback inhibition, properties of allosteric enzymes, allosteric control of central pathways, and covalent modification of enzymes.

With Chapter 8 we turn again to a strictly microbiological process, bacterial fermentation. The individual topics discussed are the following: alcohol fermentation, lactate fermentation, butyrate and butanol-acetone fermentation, mixed acid and butanediol fermentation, propionate and succinate fermentation, methane and acetate fermentation, sulfide fermentation (desulfurication', and fermentation of nitrogenous compounds (single amino acids, mixtures of amino acids and heterocyclic compounds).

Chapter 9 is devoted to chemolithotrophic and phototrophic metabolism, and the last chapter, Chapter 10, to a clear and concise summary of the fixation of molecular nitrogen by bacteria.

For this book to be useful the reader has to have a firm basic knowledge of both general biochemistry and microbiology. It is a good reference work which presents, in a concise form, all metabolic features specific to bacteria, but seems to have some didactic shortcomings. The reviewer has the feeling that the author was not quite successful in his original attempt to present first what could be called "basic metabolism" with *E. coli* as the experimental object, and then to enlarge his scope in a concentric way. This strategy of treating the material involves a lot of repetitions and inconsistencies in the logical structure of the lay-out of the material and finally leads to some confusion in deciding how the different metabolic pathways, whose descriptions are scattered all over the various chapters, are integrated into the general network of metabolism as a whole. In the reviewer's opinion a more conventional "linear" approach to describe bacterial metabolism would have been more successful. Of course, this is not meant to say that the book is of little use. It serves as a very helpful supplement for both the teacher and the student engaged in the study of the metabolism of bacteria. The individual chapters are written in a very clear way, with a lot of extremely useful information and due emphasis on the very essence of the problem under discussion. It is just the overall lay-out of the book the reviewer can hardly agree with from a didactic point of view.

F. SOLYMOSY

Transition Metals in Biochemistry, by Arthur S. Brill. Springer-Verlag, Berlin—Heidelberg—New York 1977. p. 186

This book consists of six chapters. The first of them deals with fundamental questions of the subject, such as definitions of transition metals, prosthetic groups in proteins, equilibrium in reactions of transition metals, functions of protein molecules containing transition metals. As regards the role played by the transition metals in the mechanism of action of proteins, the author poses the following six fundamental questions: What is the electronic configuration of the metal ion and what is the spin state? To how many

atoms is the metal ion bonded, and how are they disposed? What is the chemical nature of each bond? How covalent are the bonds? What are the bond lengths? How do the answers to the above questions change as the protein carries out its function? — In the following the author summarizes the various physico-chemical methods suitable for the study of metal complexes, pointing out how each method can answer some of the above six questions. In the last part of the first chapter some aspects of the role of the polypeptide molecule in the formation of the metal binding site and in the functioning of proteins containing transition metal ions are discussed.

In the second chapter ("Metal Coordination in Proteins") the author deals with the role of the functional groups of proteins in the metal binding, and with the features of metal ion coordination in several well studied proteins, such as insulin, carboxypeptidase, myoglobin, hemoglobin, myohemerithrin and hemerithrin, cytochrom c, cytochrom b_5, copper and zinc superoxide dismutases, rubredoxin, ferredoxin, and high potential iron-sulfur proteins.

The third chapter ("Copper") is a summary of the experimental results and the theoretical explanations of data concerning the optical and EPR spectral properties of copper complexes, including the copper peptides, the "blue"-type and the "non-blue"-type copper proteins.

In the fourth chapter ("Heme Iron") the properties of the complexes of iron with proteins containing heme prosthetic group are described. The valence state of iron in these complexes and its experimental determination, the spin states of iron, the optical and EPR spectral properties of these hemeproteins are particularly discussed. The photodissociation of the carbon monoxide-containing hemeproteins and the kinetics of the recombination of the complexes are also treated.

The fundamental physico-chemical characteristics of the iron-sulfur proteins and the molybdenum-containing enzymes are briefly summarized in the fifth chapter ("Non-heme Iron and Molybdenum").

In the last chapter ("Electronic Structure and Properties") some fundamental questions concerning atomic orbitals, spin states, electronic structure in the transition metal ions, properties of ligands of biological importance, molecular orbitals, light absorption, interaction of transition metal ions with an applied magnetic field, magnetic interaction of metal electrons with nuclei in the coordination sphere, and optical activity, are interpreted in terms of quantum chemistry.

The book is not a review of a field of inorganic biochemistry. Its great advantage is that the author, a well-known, excellent specialist of coordination chemistry, did not want to show and discuss in an equal measure all of the important results in this extremely broad field of modern biochemistry; only some selected problems are treated in depth. The selection seems to be very fortunate as regards the understanding of the physico-chemical properties and biological functions of the proteins containing transition metal ions. An additional great value of the book is that it contains a long list of references and the reader can easily find data and descriptions on the problems treated or only briefly mentioned in the text.

L. Boross

Functions of Glutathione in Liver and Kidney by H. Sies and Q. Wendel (eds), Springer-Verlag, Berlin—Heidelberg—New York 1978. pp. XIII + 212.

This volume in the Proceedings in Life Sciences series is actually the collection of the papers presented at the 25th Konferenz der Gesellschaft für Biologische Chemie held at Schloss Reisenburg, Germany, July 8—11, 1978. For the sake of rapid publication the editors had to refrain from including the discussions. The book is divided into four chapters.

Chapter 1 contains papers on the regulation of the glutathione (GSH) level in liver. N. Tateishi and T. Higashi show that the well-known rapid metabolic turnover of GSH in rat liver is due to the existence of a so-called "labile" pool of GSH, with a half life of 1.7 hours, in addition to another pool with a half life of 28.5 hours. This "labile" pool of GSH functions as a reservoir of cysteine. In hepatocytes, isolated in the

presence of EGTA, methionine and homocysteine have a beneficial effect in maintaining GSH levels. H. A. Krebs et al. suggest that this is due to a gradual release of cysteine via the cystathionine pathway, needed for the resynthesis of GSH. The importance of the cystathionine pathway in maintaining the GSH level in isolated hepatocytes is further analyzed by D. J. Reed and P. W. Beatty. Elevation of liver gamma-glutamyltranspeptidase in late pregnancy in the mouse is regarded by T. Higashi and N. Tateishi as a process that probably ensures an adequate supply of cysteine to meet the increasing demands of the pups for a proper level of cysteine. Two contributions dealing with glutathione transport, one by G. M. Bartoli et al. about glutathione efflux from perfused rat liver and its relation to glutathione uptake by the kidney, and another one by R. Hahn and W. Oberrauch about the unidirectional transport of GSH in rat liver and its metabolization in the extracellular space conclude this chapter.

Chapter 2 entitled "The Role of gamma-Glutamyl-Transferase in Glutathione Turnover" starts with a fine review by A. Meister on the current status of the gamma-glutamyl cycle. Most of the 69 papers referred to have been written by the author and his co-workers, an enviable achievement. The topology and function of renal gamma-glutamyltranspeptidase (in this book the terms gamma-glutamyl-transferase (E.C. 2.3.2.2.) and gamma-glutamyl-transpeptidase are used interchangeably) is discussed by S. Silbernagel and co-workers. They have used a very sophisticated method, micro-perfusion of single rat nephrons in vivo et in situ, to follow the fate of GSH and some of its analogs and derivatives. N. P. Curthoys and co-workers, in their paper on the membrane association and physiological function of rat renal gamma-glutamyl-transpeptidase, point out that their results are inconsistent with the hypothesis that this enzyme participates in amino acid transport via the gamma-glutamyl cycle, but instead support the proposal that the principal function of gamma-glutamyl-transpeptidase is the extracellular degradation of glutathione. J. S. Elce, from his studies on the active site of gamma-glutamyl-transferase, concludes that a lysine residue is important for enzyme activity and that in the gamma-glutamyl — enzyme intermediate the gamma-glutamyl residue must be attached to a second lysine residue by an amide linkage. This chapter ends with a contribution by F. H. Leibach et al., suggesting that GSH is intimately associated with transport processes in the kidney.

Chapter 3 entitled "Hydroperoxide and Disulfide Metabolism" is introduced by an overview of hydroperoxide metabolism, written by B. Chance and co-workers. In a general diagram the sources and sinks for oxygen reduction products in the mitochondrial, cytosolic, and peroxisomal spaces are clearly indicated. The generation of the oxygen intermediates O^- and H_2O_2 may lead to the production of the unstable and reactive oxygen species $HO\cdot$ and $^1O_2$, which, in turn, are able to initiate a free radical chain reaction that leads to lipid and organic peroxide formation. The factors responsible for maintaining the normal redox state of the cell are discussed in detail, with special emphasis on the role of oxidized glutathione (GSSG) release. This process points to the importance of the functioning of glutathione peroxidase. Some properties of the selenium moiety of glutathione peroxidase and of non-selenium-dependent glutathione peroxidase are described in detail by A. Wendel et al. and R. F. Burk and R. A. Lawrence, respectively. Some characteristics of GSSG and glutathione-S-conjugate release from perfused rat liver are analyzed by H. Sies and co-workers. From his studies of the reduction of diazenes and neutral hydroperoxides (recognized thiol oxidants) by rat liver mitochondria P. C. Jocelyn concludes that most probably it is the glutathione redox system that is chiefly responsible for the reduction of thiol oxidants and that both the penetration of the oxidant and the regeneration of GSH from GSSG are energy dependent processes. A further use of a diazene (diamide) allowed N. Siliprandi and co-workers to analyze the effect of oxidation of glutathione and membrane thiol groups on mitochondrial functions with special emphasis on Ca^{++} and Mg^{++} transport. This chapter concludes with a paper by B. Mannervik and K. Axelsson on the roles of glutathione, thioltransferase, and glutathione reductase in the scission of sulfur—sulfur bonds. Their main conclusion is

that the role of GSH in the scission of sulfur—sulfur bonds is catalytic.

The fourth and last chapter is concerned with pharmacological and toxicological aspects. The presentations by W. B. Jakoby and by S. Orrenius and D. P. Jones have rightly been placed at the head of the series of papers included in this chapter, because they both are of general interest and have the character of a review, even if the latter includes a number of individual experimental results. The lecture by W. B. Jakoby is an extremely lucid short summary of the functions of glutathione transferases in detoxification. The point the author makes is that these enzymes appear to catalyze all reactions that would be expected of GSH acting as a nucleophile, providing that the electrophilic substrate is bound to the enzyme. A scheme clearly summarizes the different aspects of this statement. The much longer presentation by S. Orrenius and D. P. Jones, dealing with the functions of glutathione in drug metabolism, equals the former one in clarity. The discussion of the role of glutathione in hepatic detoxication reactions is centered on three functions of glutathione: GSH as a reductant (1) is responsible for peroxide reduction, as a cofactor (2) it takes part in aldehyde and alfa-ketoaldehyde oxidation, and as a nucleophile (3) it acts in drug conjugation. Some very interesting details concerning lipid peroxidation induced by ethanol and halogenated hydrocarbons in vivo are discussed by H. Kappus et al., drug-induced lipid peroxidation in mouse liver by A. Wendel et al., cellular functions in glutathione-depleted hepatocytes by J. Högberg et al., glutathione and glutathione conjugate metabolism in isolated liver and kidney cells by D. P. Jones et al., and glutathione-requiring enzymes in the metabolism of prostaglandin endoperoxides by E. Christ-Hazelhof and D. H. Nugteren.

The papers presented in this volume contain, in most instances, brand-new information and the reviews introducing the individual chapters give a fine coverage of the research field concerned. In this way the book fulfills a dual purpose: it serves as a source of recent information for the active specialist and gives, at the same time, a comprehensive overview of our present state of knowledge of the functions of glutathione in liver and kidney. Therefore both experts and beginners in the field will have the feeling the book has been meant just for them.

F. Solymosy

Snake venoms. Handbook of Experimental Pharmacology, Vol. 52, by Lee, C.-Y. (ed.), Springer Verlag, Berlin—Heidelberg—New York 1979, 1130 pages, 208 Figures

Snake venoms are beneficial not only to their hosts in defence and attack but, interestingly enough, have contributed to the progress of numerous fields of biological research, such as investigations of acetyl choline receptors, discovery of bradykinin and studies of biological membranes and transport processes.

The volume contains 28 chapters contributed by 34 authors.

In the first part according to the classical way of presentation, historical aspects, the taxonomy of snakes, their distribution on the Earth and, at last, the structure and function of the poison gland are discussed with honourable brevity.

The second part from Chapter 4 to Chapter 9 deals with the chemistry and biochemistry of snake venoms. Chapter 4 summarizes present-day knowledge of the isolation of venom enzymes and the properties of their 17 representatives so far studied in more detail. Enzymes of this group are, for example, a phosphodiesterase, phospholipases and others that have successfully been applied for analytical or other purprses in numerous fields of biochemistry (e.g. nucleic acid sequencing, phospholipid research, etc.). Chapter 5 discusses the chemistry of toxins. Up to now, the amino acid sequence of more than one hundred of them has been elucidated and, in addition, exploration of their three dimensional structure is promising for understanding their effects on synapses and thus getting closer to the functional build-up of synapses. By a chemical-paleogenetical analysis of the amino acid sequences, attempts were made to reveal the phylogenetic development of toxins (Chapter 7). The last two chapters of Part Two discuss the importance of the nerve growth factor and that of the non-protein components of snake venoms.

The nine Chapters of Part Three deal with the most thoroughly studied problems in this field, the pharmacological effects of venoms. In this particular field physiological research, with these substances has led to several fundamental observations especially as far as acetyl choline receptors and ionic distribution are concerned.

The specific haemolytic, haemorrhagic, necrotizing, oedema producing and cardiovascular effects of the toxins are the subject of four chapters. Chapter 17 may be of special interest to laboratory workers, as it deals with the application of toxins as experimental tools. Some of these possibilities (Chapter 18) are routinely used in clinical diagnosis to measure blood coagulation.

Part Four, the last one, deals with immunological and clinical problems: Chapters 19—22 cover the immunological and complement properties of venoms and Chapter 23 delineates the methods of vaccina production. The last Chapters describe some symptoms of snake bite together with the appropriate therapy.

The book offers a detailed compilation of very high level. It provides useful help and information to all those researchers who deal with either the theoretical or practical aspects of snake venoms.

P. ELŐDI

Oncogenesis and Herpesviruses III. Proceedings of the Third International Symposium on Oncogenesis and Herpesviruses held in Cambridge, Mass., USA 25—29 July, 1977 by G. de. Thé, W. Henle and F. Rapp (eds), technical editor for IARS W. Davis, IARS Scientific Publication No. 24., International Agency for Research on Cancer, Lyon 1978, pp. 1102

This proceedings published in two volumes contains 125 papers forming 8 main topics. Each one is introduced by one or more introductory review papers and concluded with a discussion summary. At the end of the 2nd volume there is a summing-up section containing the provisional classification of herpesviruses and a summary and indication of priorities in the research field of oncogenic herpesviruses. The authors' index and the list of scientific publications of IARS are a useful guide for the reader.

The first topic of Part One is devoted to the structure of herpesvirus DNA and its association with host cell DNA. The introductory review article by H. zur Hausen and co-workers on Epstein—Barr virus genomes and their biological functions is followed by 12 papers dealing with herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) Epstein—Barr virus (EBV), cytomegalovirus (CMV) and Aujeszky's disease virus (ADV). The discussion is summarized by N. M. Wilkie who points out that cells transformed by herpesviruses may contain the viral DNA in plasmid form instead of being integrated. It cannot be excluded, however, that a small subgenomic part of herpesvirus DNA is inserted because of a lack of sufficiently sensitive hybridization techniques.

Viral antigens and strain differences of herpesviruses form the second chapter. This is introduced by two review articles. The first one by P. G. Spear and co-workers clearly summarizes available data about the roles of glycosylated and non-glycosylated proteins specified by herpesviruses in the morphogenesis and function of the virion. This is followed by Deinhardt and Wolf's paper focussed on similarities and differences amongst various herpesviruses. They briefly describe the three subfamilies of herpesviruses and give a detailed description of Gammaherpesviridae of primates. The following 10 papers are classified into four groups in N. Frenkel's discussion summary: (1) Antigenic relatedness of herpesviruses belonging to subfamilies α, β and γ. It has been reported that the nucleocapsids of HSV-1, HSV-2, EBV, EHV and Lucké herpesvirus share a common antigen, which, surprisingly, is also present in the mammalian oncorna virion. This conflicting result, however, needs further clarification. (2) Purification and characterization of viral antigens. A two-dimensional PAGE electrophoresis is described for the separation and isolation of viral antigens which, after being subjected to this procedure, retain their biological activity. (3) Tumor-associated viral antigens.

The last two chapters of Volume 1 and the first one in Volume 2 are devoted to one of the most intriguing areas of oncogenic herpesviruses under the heading of "Cell–Virus Interaction".

Molecular events in permissive and semi-permissive cycles form the 3rd chapter of Volume 1. Roizman and Morse's review article is focussed mainly on HSV-1 and HSV-2. This is an excellent source of information concerning the synthesis and regulation of herpesvirus polypeptides. The 12 papers presented in this section describe recent progress in our understanding of molecular processes involved in the replication of CMV and EBV. Significant progress has been achieved in genetic mapping of HSV, which is of great value.

The fourth chapter of Volume 1 is focussed on the integration and expression of the viral genome. The introductory paper by F. Rapp and E. J. Shillito summarizes our current knowledge of the various cell systems so far successfully transformed by different herpesviruses. The second introductory review of this chapter is devoted to the transformation of lymphoid cells by herpesviruses. This important descriptive paper by K. Nilsson presents the available data on lymphoid cells transformed by EBV, three primate herpesviruses and Marek's disease virus. A method for detecting HSV DNA sequences in transformed cells is reported. The expression and regulation of thymidine kinase gene of HSV are the topics of several papers. Lymphoid cells transformed by EBV are further characterized and classified into two main groups. A comparison of transforming and non-transforming EBV DNA indicates that the transforming one possesses some unique sequences. A study of the regulation of the expression of EBV genes in somatic cell hybrids is also presented.

The first chapter of the second volume is focussed on transformation and properties of transformed cells. The introductory paper by B. Hampar and A. L. Boyd is on the interaction of oncornaviruses and herpesviruses. They observed that Balb/c cells transformed by UV-inactivated HSV start to produce xenotropic type-C virus but not ecotropic virus. This virus can be detected by sensitive cell systems only. The following 9 papers describe some important characteristics of cells transformed by CMV, EBV and MDV. A new approach for characterization of haematopoietic cell lines is also presented.

The next chapter which is devoted to the host response to herpesvirus infection is introduced by A. J. Nahmias and R. B. Ashman. Presently available data on the immunology of primary and recurrent herpesvirus infection are summarized. This is more stimulating then conclusive. Unfortunately this paper does not contain some of the well documented results concerning Marek's disease. The next introductory review by J. G. Stevens is on the persistent, chronic and latent infection of herpesviruses. This paper is confined to HSV, EBV and Lucké herpesvirus. An intriguing unifying theory on the pathogenesis of infectious mononucleosis (IM), Burkit's lymphoma (BL) and nasopharyngeal carcinoma (NPC) is presented by J. S. Pagano and G. F. Okasinski. The following 14 papers describe various aspects of immunoresponses elicited by herpesvirus infection including EBV CMV and VZV. The importence of immunoresponse with respect to persistance, reactivation and oncogenesis in man awaits further confirmation. All this is summarized by A. S. Evans.

The next topic is on comparative host response to herpesvirus associated tumors. The introductory paper by W. Henle and G. Henle compares immune responses and viral markers in herpesvirus associated carcinomas. G. Klein's excellent review in which humoral and cell-mediated responses and virus markers in herpesvirus-associated lymphomas are compared mainly concerns the EBV systems. The three-step-mechanism of BL development is discussed in the light of present knowledge. The following 13 papers deal with quite different host-herpesvirus systems including somatic cell hybrids of EBV, Lucké tumors, Marek's disease and HSV.

The last but one topic of Volume 2 contains our past and most recent knowledge of the role of co-factors and prospects for control of herpesvirus-related diseases. G. de-Thé's paper is about the co-carcinogenic events in herpesvirus oncogenesis. The future prospects and the need for control of human herpesvirus-associated conditions are discussed. A useful account of our present knowledge of prospects for control of human herpesvirus-related diseases is presented by T. C. Merigan and K. H. Rand. In the second review paper W. Plowright gives an account of the presently available

herpesvirus vaccines for animals. The advantages disadvantages of live modified and inactivated vaccines are critically evaluated. The following 13 papers contain the most recent results in this field.

The last chapter of this volume contains a provisional classification of herpesviruses by B. Roizman and a summary and future prospects in the field of Marek's disease, primate oncogenic herpesviruses, herpes simplex virus, Lucké tumor herpesvirus and Epstein—Barr virus.

To sum up, this is a worthy successor to the first two volumes of this series in maintaining its uniqueness and high standard of presentation. The excellent editorial work is greatly acknowledged. The Proceedings of the Third International Symposium on Oncogenesis and Herpesviruses was meant to serve as a textbook in this rapidly growing field and this goal has been reached. It is highly recommended to specialists and to all those whose activity is connected with animal and human malignancies.

B. Lomniczi

Carcinogenic Risks. Strategies for Intervention by W. Davis and C. Rosenfeld (eds), International Agency for Research on Cancer, Lyon 1979. pp. XIX + 283

This volume contains the proceedings of a symposium organized by the International Agency for Research on Cancer (IARC) and l'Institut National de la Santé et de la Recherche Médicale (INSERM), held at the headquarters of the IARC, Lyon, France, November 30 to December 2, 1977. As such it represents IARC Scientific Publications No. 25 and INSERM Symposia Series Vol. 74.

The recognition that about 80 to 90% of cancers are directly or indirectly related to the environment prompted that Agency to organize a symposium whose major task should be to provide a sound basis for regulatory decisions to be made in the field of environmental carcinogenesis. This emphasis on the environmental aspects of the problem is duely reflected in the topics dealt with in the individual parts of the book.

The first part is about smoking and cancer. The alarming data presented by P. Denoix concerning the correlation between smoking and lung cancer set the stage for the proper appreciation of the anti-smoking legislation in Finland as reported by O. Elo.

The second part is concerned with occupational risks. Bladder cancer hazard affecting workers in the British dye stuffs, chemical and rubber industries, is reviewed from a historical point of view by H. G. Parkes. Asbestos as a cancer hazard is considered by M. L. Newhouse and also by A. A. Cross, trichloroethylene by N. N. Page. This part concludes with some thaughts by D. B. Douglas about implications of health and safety legislation.

The third part deals with food additives, drugs and pesticides. Regulation of carcinogenic food additives and drugs in the United States is summarized by R. R. Bates, and an approach of the European communities to the assessment of the carcinogenic risk of environmental chemicals, in particular artificial colorings, is put forward by P. Bordeau et al. A separate paper is devoted to aflatoxin (C. A. Linsell), which at least contains some tables to support the lecturer's conclusions. A short report by A. Somogyi on residues of carcinogenic animal drugs in food is followed by a more extensive presentation by F. Coustou about the carcinogenic risk of products used in the pharmaceutical and related industries. Asbestos, vinyl chloride, dyestuffs, artificial sweeteners and aflatoxin are given special consideration. The scheme presented for the identification of carcinogenic substances is commonplace. Pesticide carcinogenesis data and regulatory approaches are reviewed by U. Saffiotti. A tabular presentation of pesticides reported in IARC Monographs (Vols 1—15) to give positive results in carcinogenicity tests adds to the informative character of the paper. This part concludes with a short lecture by J. Sugár and co-workers about the possible carcinogenicity of the herbicide Buvinol.

The real difficulty scientists and legislators have to face when dealing with environmental carcinogenesis is most clearly indicated by what the presentations about measurement of carcinogenic risk are able to tell us. This is very little, indeed. The problems inherent in relating, even if roughly, doses of radiation to those of chemical mutagens, as proposed by R. Latarjet, in the use of

laboratory animals for assessing carcinogenic risk for man (D. P. Rall), or in the estimation of thresholds for chemical carcinogens (R. Truhaut) are grave and difficult to surpass. As shown by B. Teichmann and T. Schramm even the terms "human carcinogen" and "animal carcinogen" give rise to confusion in the minds of non-experts.

The last part is about strategies for intervention. It is introduced by a comment of M. A. Schneiderman about alfa, beta-mannitone, an incompletely characterized material made from old automobile tires by a secret process. It is impossible not to notice that this comment was placed at the head of the series of papers in this part in order to raise the feelings of the participants against manufacturers of products of obscure origin and composition before turning to discussions of strategies for intervention. A very clever move. The subsequent lectures are concerned with legal forms of intervention against carcinogenic risks (A. C. Kiss), the setup of the Clearinghouse for Environmental Carcinogens of the National Cancer Institute (A. L. Brown), and techniques in intervention against carcinogenic hazards (P. Westerholm).

This volume concludes with some recommendations to IARC and a summing-up by Sir Richard Doll.

The book will hardly appeal to pure biochemists because it does not deal directly with biochemistry. It shows clearly, however, how difficult it is to work out and get through regulatory measures concerning environmental carcinogenesis, even if they are based on irrevocable experimental facts.

F. SOLYMOSY

Immunity to Viruses by H. Fraenkel-Conrat and R. R. Wagner (eds). In: Comprehensive Virology, Vol. 15, 1979. p. 293. Plenum Press, New York and London 1979

This volume is devoted to an important aspect of virus–host interactions, namely to immunological reactions to viruses. *Immune responses, Immune tolerance and viruses* (M. B. A. Oldstone) reviews the role of cell populations of the immune system and their interactions in generating antiviral immune responses in the presence of virus. The overwhelming evidence indicates that immune responses accompany most, if not all, viral infections. Immunologically competent cells may also be infected by viruses, and these can persist in them during the animal's life. There are several mechanisms whereby viruses can persist and escape the host's immune response, but classical immunological tolerance does not occur. *Interaction of viruses with neutralizing antibodies* (B. Mandel). The interaction of virus and antibody is a complex phenomenon. Complexity derives from the fact that both virus and antibody represent families of related but variable components. Therefore both the nature of the components and mechanisms of neutralization are discussed in detail. The *in vitro* study of viral neutralization represents a "probe" for understanding the *in vivo* mechanism of the humoral defence system. A current problem related to the immune response is the recognition of unsuccessful virus–antibody interactions in the form of infectious virus–antibody complexes. These will cause a pathological syndrome now classified as immune complex diseases.

Humoral immunity to viruses (N. R. Cooper) focuses on humoral defence mechanisms that function to neutralize and inactivate viruses. Specific and nonspecific, antibody- and complement-dependent and -independent mechanisms resident in the plasma are examined. Antibody can alone neutralize many viruses non-destructively in a number of different ways, while complement, activated by immune complexes is able to amplify the effect of viral neutralization by various mechanisms. There are viruses e.g. the lipid containing ones, which found to be neutralized by complement alone in the complete absence of antibody. *Cellular immune response to viruses and the biological role of polymorphic major transplantation antigens* (R. M. Zinkernagel). This chapter outlines the main characteristics of the MHC (major histocompatibility gene complex) restriction of T cells that perform a critical biological function in the immune response including the response to virus infections. MHC of mice has been designated H-2. The antigen products of two subregions, H-2K and H-2D, participate in the murine immune response to viruses. The general rule

emerging is that T cells generated to kill virus infected target cells can do so only when the viral antigens appear on cells whose H-2 matches the H-2 type of the T cell donor. All T cell functions are H-2 restricted in mice.

Interferons (E. De Maeyer and J. De Maeyer-Guignard) gives a summary of interferons, a group of vertebrate glycoproteins which exert a broad antiviral activity in the species they derive from. Synthesis, molecular characterization, mechanisms of antiviral activity, interaction with the immune system and antitumor and cell multiplication effects of interferons are discussed.

B. LOMNICZI

Structure and Assembly. Comprehensive Virology 13: by H. Fraenkel-Conrat and R. R. Wagner (eds), Plenum, N. Y. 1979, pp. 672

"The time seems ripe for a critical compendium of that segment of the biological universe we call viruses" states the Foreword to Vol. 13 of Comprehensive Virology. We might add that time is especially adequate to give incisive and thorough reports on the latest developments in the field of nucleotide and aminoacid sequencing of viral macromolecules as well as of their assembly to virions.

Detailed descriptions of the best known systems, Adenovirus structural proteins, Adeno-SV 40 hybrids, Genetic control of complex bacteriophage assembly are presented in the sparsely, but well illustrated book. A special feature of the volume is a multicolor foldout showing the first complete nucleotide sequence of a viral RNA (MS 2 bacteriophage).

The aim of the book is to provide a comprehensive treatise on the latest methodologies of gene sequencing and recombinant DNA technology, which has triggered a geunine information explosion during the last few years. We should add that the same Chapters convince us that sequence analysis alone is not enough to work out mechanisms and control of replication, translation and transcription. In conjunction with other studies it is, however, an invaluable aid to interpreting results of genetic analysis and other (molecular) biological experiments.

Other Chapters include "Structure and function of bacteriophages" and "Structure and assembly of viral membranes", the latter providing models for approaching problems of macromolecular assembly and morphogenesis in higher organisms (with clear implications both for basic developmental biology and for eventual understanding of congenital or environmentally induced malformations).

This book is a definitive reference source of data for scientists (and postdoctoral students) in medicine, biochemistry, cancer research, molecular and cellular biology, a valuable collection of information to anyone interested in viruses or transformation as a model for studying tumorigenesis or using these viruses as cloning vehicles.

E. DUDA

Methods in Membrane Biology, Vol. 6. by E. D. Korn (editor), Plenum Press, New York 1976, pp. 248 + XVII.

Volume 6 of this valuable methodological series contains four chapters. In Chapter 1 H. Beaufay and A. Amar-Costesec (Brussels) discuss the basic techniques of cell fractionation. The authors present various methods for cell- and tissue homogenization ,describe the physical basis and practical methods of differential centrifugation, and summarize the enzymatic characterization of the cell fractions obtained. Chapters 2–4 discuss the properties of bacterial membranes. In Chapter 2 (M. R. J. Salton, New York) describes the methods for the isolation and characterization of membranes from Gram positive and Gram negative bacteria. Chapter 3 (D. F. Silbert, St. Louis) deals with the problem of membrane biogenesis, based on results obtained with *Escherichia coli* K 12 a mutant defective in fatty acid biosynthesis' Chapter 4 (work of D. L. Oxender and S. C.. Quay, Ann Arbor) presents methods for the isolation and characterization of bacterial membrane binding proteins. The method of osmotic shock is described in detail, followed by the presentation of binding proteins for amino acids, sugars, ions, and vitamins. Favourable assay conditions for investigating protein-ligand interactions are also presented.

All the four chapters of this volume contain a great deal of valuable informations for researchers working in the field of membrane biology.

G. GÁRDOS

Grundlagen der Organischen Chemie by Joachim Buddrus 754 pages, Walter de Gruyter, Berlin–New York, 1980

According to the author, Joachim Buddrus (Institute for Spectrometry and Applied Spectroscopy, Dortmund, F. R. G.), this book is intended to serve as an introductory, basic text in organic chemistry. About writing basic texts, however, two questions emerge: (1) where to start and (2) when to finish? The answer to both questions depends on for whom the book is written. In the case of Buddrus' text, unfortunately, this remains under a point:

1. The author deals with atomic and molecular orbitals, sigmatropic rearrangements, etc., without making reference to the Schrödinger equation. This is rather unfortunate for a basic text even in organic chemistry, written for any kind of students.

2. Much the same can be said about the arbitrary deletions of the book: though a detailed description of peptides and proteins is presented (including discussion of sequence analysis by mass spectrometry, too), the reader can find nothing about nucleic acids or the purine and pyrimidine bases.

Turning from the missing to the existing part of the book, its general build up resembles those of the old and renowned German texts in organic chemistry. Most of the 25 chapters deal with different groups of organic compounds. Some are focused at various types of reactions in organic chemistry, such as polymerization, nucleophilic substitution, elimination and pericyclic reactions. There is a chapter on basic stereochemistry, too. Throughout the book special emphasis is given to spectroscopic methods recently in use in organic chemistry.

The text is written in a good style and the book is presented in a nice and handy form. The figures are clear and simple. The table of contents is a bit more detailed than necessary (19 pages). 450 problems with solutions help the student to check this knowledge.

Altogether this textbook is somewhat expensive for its value.

H. HAJDU

Zell- und Gewehekulturen by John Paul. Translated by Sigrid and Rainer Maurer. Walter de Gruyter, Berlin—New York, 1980

This book originally written in English and reprinted five times, is one of the most widely handbooks among all those who apply tissue culture methods in their everyday research work. Now it came out in German, translated by Sigrid and Rainer Maurer. During the last two decades this has not been the only book designed to introduce and to popularize tissue culture methods in biological research (see books written by Willmer or Patterson, etc.), but this is the only one which was able to remain so reasonably proportioned in its chapters as it was in the first edition. At the same time, the author was able to keep up with the latest results of this method without giving up fundamental parts published in earlier issues. The book is useful not only to those who are experts in dealing with this method but provides excellent possibilities also for beginners who want to get acquianted with tissue culture methods. Besides the detailed technical descriptions the author summarizes the practical applications of tissue culture. These show that cell and tissue culture techniques have become essential for the exploration of a number of biological problems.

After a short historical survey which covers the main phases in the development of the method, the author presents the principles of cultivation, dealing with the cell and its environment, the growth and metabolism of cultured cells and natural and defined media.

Part III, one of the most useful chapters from a technical point of view, includes preparation of materials, sterilization procedures, aseptic techniques and design and equipment of a tissue culture laboratory. This could serve as a separate manual for technical aids in a tissue culture laboratory.

Chapter IV, which includes four headings, gives accurate and detailed information

about the special tissue culture techniques including the preparation of primary and secondary cultures, organ and disaggregated cultures, the basic requirements of establishing cell lines and developing suspension cultures, advantages of largescale cultivation over other cultivation systems, etc. Furthermore, this chapter summarizes the basic principles and the technical demands for the cultivation of invertebrate and plant tissues. Data on in vivo cultivation or transplantation and a short survey about the preservation, storage and transport of living tissues and cells are added to this chapter.

In Chapter V the author summarizes special applications of cell and tissue culture methods. Besides the most widely used morphological and quantitative studies detailed information about tissue cultures used in biomedical research, in genetics, virology and cancer research supplement this part.

The appendix contains a great deal of practical information, among others composition and preparation of the most often used chemically defined media. The addresses of companies and transporters interested in the production of glass and plastic wares, nutrient media and sera suitable for the maintenance of living cells in vitro etc., help freshmen to start work.

In the addendum a list of cell lines available with relevant scientific information such as their species origin, derivation, ATCC number, etc., and the list of journals specially designed to publish papers about in vitro works, furthermore books and monographs published about this method make this handbook perfect.

A. GYÉVAI

Strategies of Microbial Life in Extreme Environments. Life Sciences Research Reports Vol. 13. by Moshe Shilo (ed.). Verlag Chemie, Weinheim, New York

The 13th volume of the series "Life Sciences Research Reports" deals with results and new aspects in the field of biochemistry, genetics and ecology of microorganisms living under extreme environment.

The book contains the reports of the Dahlem Workshop held in 1978 on the above subject. It has four main chapters: 1. Life at low water activities; 2. Oxygen toxicity; 3. Life under conditions of low nutrient concentrations; 4. Life at high temperatures.

Among the authors of 29 reports we find the most prominent researchers of this subject. They look for the evolutionary strategies for adaptation in natural and man-made extreme environments, analyse the mechanisms underlying adaptation to stress conditions and attempt to consider possible directions for future research in this field.

Two of the four main chapters of the book, Chapters 1 and 3 deal with ecological problems mainly analyzing the effect of environmental factors on adaptational processes. These chapters lack a discussion of biochemical and genetical problems, so they are somewhat unsophisticated.

The presence of Chapter 2 in the book is slightly surprising. Oxygen toxicity has not been included among extreme environmental factors in the literature before, but, as revealed from reports presented here, the existence of connection cannot be denied.

The authors investigate the effect of oxygen and its derivatives on various anaerobic and aerobic organism. In this chapter the point is shifted to biochemistry and physiology. Many fascinating experimental data and theoretical comments are presented on the role of oxygen in the living cell.

Chapter 4 is the most homogeneous and, at the same time, the most versatile one, where we can find reports on the evolution and ecology as well as on the biochemistry and genetics of thermophilic microorganisms. The authors give a comprehensive summary of the related fields in detailed reports containing about 30 references each.

Despite some insufficiencies mentioned above the book gives a comprehensive summary of the propertis of life in extreme environments. The great number of references helps newcomers in rapid orientation.

M. KÁLMÁN

Mammalian Endogenous Peroxidases as Cellular Markers and as Biosynthetic Endpoints of Hormone-Mediated Activity: Viewpoint from Cytochemistry by W. A. Anderson, Y.-H. Kang and S. Mohla. Progress in

Histochemistry and Cytochemistry, Vol. 11., No. 4. Gustav Fischer Verlag, Stuttgart, New York, pp. 27, 14 Figures, 3 Tables

The title accurately reflects the topic of this monograph.

The introduction describes how the 3,3'-diaminobenzidine tetrahydrochloride technique has facilitated both the microscopic and the electron microscopic investigation of intra- and extracellular peroxidases (P-ases). It becomes clear that the P-ases, such as horseradish P-ase, known for a long time, can be employed as excellent markers in electron microscopy and in immunocytochemistry (e.g. RIA) for the detection, localization and the quantitative measurement of P-ases or for the delineation of the interstitial spaces and determination of membrane permeability. Thus, the P-ases have become very important tools in modern research. At the same time, a fuller understanding of the role of the endogeneous P-ases has recently been achieved. Of the endogenous P-ases, the authors first deal in detail with the properties of lactoperoxidase (LP-ase). They discuss the hormone dependence of LP-ase formation, the nature of the enzyme marker, and the hormonal correlations of experimentally induced mammary tumours. Some genetic aspects of the synthesis of LP-ase are also known. Both here and in connection with other, exogenous P-ases (those secreted from the acini), the authors deal with their assumed roles; of these, the antimicrobial and antiviroid roles are of greatest importance.

A hormone-controlled P-ase, of similar importance from the point of view of biosynthesis, is thyroid peroxidase (TP-ase). The main function of TP-ase is thyroxine production and thyroxine binding to protein. In addition to the P-ases already mentioned, the monograph also discusses the Kupffer cell (macrophages, white blood cells) endogenous P-ases and uterus P-ase. The endogenous P-ases are important enzymes which both intra- and extracellular activities. They occur almost everywhere in animal tissues, and have well defined functions. Their synthesis is controlled by hormones, and they are therefore good markers of cell dynamism. As already mentioned, they may be utilized for microscopic and electron microscopic localization, and for many cytological purposes.

The clear Tables, excellent microscopic and electron microscopic pictures and the subject index make the monograph a lasting review of this topic. There are almost 90 literature references, arranged alphabetically according to the names of the first authors, and the titles of the papers referred to are also given.

B. MATKOVICS

A Handbook of Radioactivity Measurement Procedures, *NCRP Report* No. 58. National Council on Radiation Protection and Measurements, Washington, D. C., 1978, pp. 506

The report was written by the NCRP Scientific Committee 18A consisting of 7 members and 14 consultants under the chairmanship of Wilfrid B. Mann.

The book is very useful for researchers working with radiolabelled compounds. It consists of eight chapters, appendices and references. The book's subtitle: "With Nuclear Data for Some Biologically Important Radionuclides" shows its general scope.

Chapter 1 is a brief introduction including basic terminology for the description of radioactive decay and the physics of the interaction of radiation with matter. Radioactive standardization and related problems are also discussed in this chapter.

Chapter 2 describes the physics of various types of detectors for radiation: ionization chambers, proportional counters, GM counters, scintillation detectors and semiconductor detectors, as well as Cerenkov counting. A summary of terms characterizing detectors, such as dead-time, decay and background corrections, is attached to the discussion of detectors.

Chapters 3 and 4 give detailed treatment of direct and indirect measurements of radioactivity. Both chapters are very useful for a great variety of applications. Chapter 5 deals with procedures for the preparation of standards and samples for counting and calibration.

Chapter 6 is an overview of application-oriented laboratory design. It presents a number of concrete assay methods and their applications. At the end of the chapter there

are a few suggestions for setting up specific or general-purpose equipment for radioactivity measurement. However, as indicated in the book, there is another report of the NCRP Scientific Committee 18B in preparation (or published already) on measurement procedures specifically concerned with medical and biological applications, that might be even more useful for users of this type of equipment.

Chapter 7 is a summary of basic statistics needed for the treatment of counting data. Chapter 8 shows the problems associated with the accuracy and precision of radioactivity measurements and underlines the importance of standards on national and intereational levels.

Appendix A (135 pages) contains a list of radionuclides and a table of nuclear decay data together with a short explanatory section. Appendix B (9 pages) is a more detailed statistical treatment of radioactive decay. The alphabetical list of references (36 pages) covers a number of applications.

The book may serve as a very useful handbook for specialists of the radioactivity field and others just using radiolabelled compounds. The most commonly used radionuclides include H-3, C-14, P-32, S-35, I-125, and only highly specialized and sophisticated work requires more "exotic" radionuclides. The book covers practically the whole spectrum of radionuclides, their physical properties and their detection and measurement, thus it may be recommended as a reference source.

K. NIKOLICS

Characterization of Protein Conformation and Function by Felix Franks (ed.), Symposium Press, London, 157 pages with 131 Figures

The volume contains lectures presented at a Chemical Society Residential School held at Wye College in September, 1977. Most of the papers deal with the basic structural units of protein folding, the stability of native conformation, the forces stabilizing protein structure, the influence of solvents on conformation and activity of proteins. Separate chapters are devoted to specialized proteins. Separate chapters are devoted to specialized proteins such as those involved in defense mechanisms, in chemical/mechanical energy transduction and in the structural organization of chromosomes. Resent advances in the research of protein transport across membranes and in the self-assembly and function of multisubunit structures are also covered. The aim of the Chemical Society Residential Schools is to update chemists in contemporary advances, the volume is therefore valuable primarily for those who want to get a concise picture of the recent progress made in areas outside their own specialty. The interested reader will find a list of further suggested readings at the end of each chapter.

L. PATTHY

Virology in Agriculture, Vol. I, Beltsville Symposia on Agricultural Research by T. O. Diener, Abacus Press, Tunbridge Wells, U. K. 1977

This 293-page volume is the first in a series of books that will report proceedings of the Annual Beltsville Symposia on Agricultural Research. The book contains 18 contributions by invited speakers (mostly American) of the first symposium that took place in May, 1976. Since the topics dealt with represent "hot spots" of virus research in agriculture both at the applied and the basic level, it is only natural that the reader will encounter some duplications of results reported not only in journals but in proceedings of other meetings as well. This, however, does not detract anything from the value of the present material because the lectures offer concise and clear descriptions of the state of art, as it were, in 1976. The content is arranged in five parts.

Molecular biology of animal and plant viruses is devoted to the biochemistry and immunogenicity of foot and mouth disease virus (FMDV) and to replication of plant viruses. The importance of knowing the molecular biology of a virus like FMDV cannot easily be exaggerated in the view that the disease is till prevalent in some countries and about 800 million doses of FMDV vaccine are produced each year. The polypeptide carrying the relevant antigenic determinants of FMDV has been identified. Thus ways of subunit vaccine production

have been opened up. The speciality of many plant viruses, the multipartite nature of their RNA genome is tackled in a mere ten pages. The main topics of the part entitled. *Virology in the service of agriculture* are: Marek's disease of chickens, bovine reproductive diseases and practical application of insect viruses. Research of the Marek's disease examplifies the best, how concentrated research effort produces quick and sound results within a decade: evaluation of research expenditures in the United States has shown that every dollar spent on Marek's disease research in the US returns 44 dollars in economic benefits. Baculoviruses represent a new kind of agent for pest control, being completely innocuous to life forms other than insects. Studies on the double-stranded RNA-containing particles found in cultivated mushrooms and plant pathogenic fungi have not lead to an unequivocal conclusion as to their biological significance let alone proof of their viral nature. Nevertheless these particles may represent an interesting stage in the evolution of genetic elements. *Approches to disease problems* brings together data on viral agents of diverse origin and biology, such as porcine enteric viruses and problems relating to immunity, recent development of bovine leukemia research, defective plant viruses and mycoplasma viruses as probes to study the biology of mycoplasmas. The final part is devoted to *nucleic acids in disease processes*. Special significance is attached to certain plasmids of phytopathogenic bacteria because plasmids carry not only genes for nitrogen fixation but also the ones that confer virulence on plants. A brief treatment is given of viroids as well, the recently recognized class of subviral plant pathogens.

The volume is not only useful to researchers interested in recent developments of virology in agriculture but proves to be a very readable text indeed.

B. LOMNICZI

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Walter de Gruyter · Berlin · New York

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1980. XI. 4. — Terjedelem: 8,75 (A/5) ív, 10 ábra

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8. VI. 1981 HU ISSN 0001—5253 Index: 26.030