FORMERLY ACTA BIOCHIMICA ET BIOPHYSICA ACADEMIAE SCIENTIARUM HUNGARICAE

Acta Biochimica et Biophysica Hungarica

VOLUME 21, NUMBERS 1–2, 1986

Akadémiai Kiadó, Budapest
ABBPAP 21 (1–2) 1–158 (1986) HU ISSN 0001-5253

Acta Biochimica et Biophysica Hungarica

a Quarterly of the Hungarian Academy of Sciences

Editors

P. ELŐDI and J. TIGYI

Managing editors

P. GERGELY and A. NIEDETZKY

Acta Biochimica et Biophysica Hungarica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1054 Budapest, Alkotmány u. 21

Subscription information

Orders should be addressed to

KULTURA Foreign Trading Company
H-1389 Budapest P.O.Box 149

or to its representatives abroad.

Acta Biochimica et Biophysica Hungarica is indexed in Current Contents

INSTRUCTION TO AUTHORS

Acta Biochimica et Biophysica Hungarica will primarily publish original and significant papers from Hungarian research institutes, universities and other laboratories. Original papers written in English in diverse fields of modern experimental biology will be considered for publication. Novel aspects of the information described in the paper should be clearly emphasized.

Manuscripts (one original and two copies) should be submitted to

Dr. P. Gergely, Department of Medical Chemistry, University Medical School, 4026 Debrecen, POB 7, Hungary *(Biochemistry)*

or

Dr. A. Nidetzky, Department of Biophysics, University Medical School, 7624 Pécs, POB 99, Hungary *(Biophysics)*.

To ensure rapid and accurate publication, the author(s) are invited to follow the instructions described below. Manuscripts which do not conform these rules will be returned.

Preparation of manuscript

Full-length papers should not exceed 10 typewritten pages on A4 size high quality paper. The complete manuscript including the space occupied by figures, tables, references, acknowledgement etc. should not exceed 15 pages.

Short communications of total length of 5 pages will also be accepted.

Acta Biochimica et Biophysica Hungarica

VOLUME 21

Akadémiai Kiadó, Budapest
1986

Acta Biochimica et Biophysica Hungarica, Volume 21, Number 1-4, 1986

CONTENTS

AUTHOR INDEX

EDITORIAL

Acta Biochimica et Biophysica Hungarica is a continuation of Acta Biochimica et Biophysica Academiae Scientiarum Hungariae, published in 1986 as its 21st volume. It is not only the title of the journal which becomes simpler; due to the new editorial policy, the publication will become quicker by the photo-offset printing. This will probably put an end to the delays occurring in the past few years with volumes published by the conventional. In 1986 the manuscripts accepted previously will be published. New manuscripts may be sent from the second part of the year, their publication is planned within 4 months following acceptance.

With the introduction of the new technique, new Instructions to Authors were prepared. It is the authors' interest that manuscripts should be carefully prepared according to the instructions. The Publisher is ready to accept high-key photos (microphotographs, etc.), but they will be reproduced by traditional printing methods.

Results of Hungarian scientists will be included in the journal primarily, however, manuscripts from invited foreign scientists are also planned to be published.

Acta Biochim. Biophys. Hung. 21(1-2), pp. 3-11 (1986)

EFFECT OF A HEME-PEPTIDE DERIVED FROM CYTOCHROME-C ON LIPID PEROXIDATION. I. EFFECTS ON BRAIN MICROSOMES

L. Vodnyánszky, A. Marton, M. Végh, A. Blázovits, F. Auth[§], A. Vértes, I. Horváth

Second Institute of Biochemistry, Semmelweis University Medical School, Budapest, Hungary, and Laboratory of Nuclear Chemistry, Eötvös University, Budapest, Hungary[§]

(Received November 30, 1984)

SUMMARY

Heme-nonapeptide inhibits NADH and NADPH dependent lipid peroxidation of brain microsomes in the presence or absence of ADP-Fe complex. The transient accumulation of lipid peroxides during NADH or NADPH dependent, ADP-Fe stimulated lipid peroxidation, is inhibited by heme-nonapeptide. Oxygen consumption of brain microsomes in the presence of NADH or NADPH is stimulated by heme-nonapeptide. Reduction of cytochrome-c and nitro-tetrazolium-blue by O_2^- generated by xanthine oxidase is inhibited by heme-nonapeptide.

INTRODUCTION

Proteolytic fragmentation of cytochrome-c yields oligopeptides containing covalently bound proto-heme. Murata et al. (1973) have demonstrated that the elevation of hepatic malondialdehyde content, induced by bleeding or vitamin E deficiency can be counteracted by parenteral administration of heme-octapeptide. These pharmacological results may be explained by an inhibitory action of heme-peptide on lipid peroxidation.

Abbreviations: CHP-9, heme-nonapeptide; SOD, superoxide dismutase; MDA, malon-dialdehyde; LOOH, lipid peroxide

Akadémiai Kiadó, Budapest

We investigated this problem in vitro on microsomes in the hope that we would obtain some information on the largely unknown mechanism of lipid peroxidation.

The recent model of lipid peroxidation has been based on the fundamental experiments of Ernster et al. (1964) who demonstrated that in the presence of NADPH the lipid peroxidation of liver microsomes is markedly stimulated by ADP-Fe complex. In a separate paper from this laboratory Venekei et al. (1985) it has been reported that heme-peptides (heme-containing, autooxidable derivatives of cytochrome-c) do not influence the ADP-Fe stimulated peroxidation of liver microsomes. Brain microsomes also exhibit enzymatic lipid peroxidation which can be stimulated by ADP-Fe as shown by Player and Horton (1981). This experimental system was applied in the present study for the investigation of the effect of heme-nonapeptide of cytochrome-c on lipid peroxidation.

MATERIALS AND METHODS

Microsomes were prepared from the brain of Wistar rats weighing 150-200 g. Whole brains were homogenized (10% w/v) in 0.25 M sucrose, pH 7.4. The homogenate was centrifuged 15 000 x g for 20 min. From the supernatant the microsomes were pelleted by centrifugation at 78 000 x g for 60 min. The pellet was washed then suspended in 0.15 M KCl (10 mg protein per ml) and stored at -20°C.

Lipid peroxidation of brain microsomes was investigated under the conditions described by Player and Horton (1981), using 50 mM Tris-maleate buffer (pH 6.8) in the presence of 1 mM potassium phosphate. The reaction mixture contained 20 µM $FeCl_3$, 2 mM ADP, 300 µM NADH or NADPH and 1 mg/ml microsomal protein. The reaction was stopped by the addition of thiobarbiturate reagent used for the photometric determination of malondialdehyde formation (Buege and Aust, 1978). Oxygen consumption was measured by a Clark oxygen electrode. The lipid peroxide content of microsomes was determined iodometrically after extraction (Buege and Aust, 1978). The activities of microsomal NADPH:cytochrome-c reductase and NADH:cytochrome-b_5 reductase enzymes were assayed by the spectrophotometric measurement of the reduction of cytochrome-c (Wermillion and Coon, 1974) and the oxidation of NADH (Rogers and Strittmatter, 1973), respectively. The degree of reduction of iron in the ADP-Fe complex under various experimental conditions was assessed by Mössbauer spectrography using a "Ranger Electronics" spectrometer. In these experiments the protein concentration was 5.8 mg/ml which was necessary to obtain measurable signals. 4 mM $^{57}FeCl_3$ and 5 mM ADP were added to the reaction mixtures and were rapidly

frozen in liquid nitrogen then measured.

Xanthine oxidase was prepared from milk by the method of Waud et al. (1975). Its specific activity was 0.4 μmole uric acid/mg protein/min when assayed in 0.1 M phosphate buffer, pH 7.8 in the presence of 2 mM EDTA, at 25°C. In the xanthine oxidase system the reduction of cytochrome-c was determined according to McCord and Fridovich (1969) and the reduction of tetrazolium-blue was measured according to Younes and Weser (1976). Protein was measured be the method of Lowry et al. (1951).

Heme-nonapeptide was obtained from Reanal Fine Chemicals (Budapest). Its purity was found at least 95% determined by paperchromatography, amino acid analysis and spectrophotometric measurement. Cytochrome-c was provided by Boehringer (Mannheim). Superoxide dismutase was purchased from Sigma (St. Louis, MO) and xanthine from Fluka (Basel). All other chemicals used were the products of Reanal (Hungary).

RESULTS

The effect of heme-nonapeptide on the NADPH and NADH dependent lipid peroxidation was examined in brain microsomes Fig. 1). The formation of malondialdehyde is generally regarded as a suitable indicator of lipid peroxidation. Heme-nonapeptide inhibited both the unstimulated and the ADP-Fe stimulated malondialdehyde formation. The inhibition was most significant when NADH and ADP-Fe were present. There was a positive correlation between the rate of malondialdehyde formation in the presence of different coenzymes and the specific activities of the respective reductase enzymes (14 nmole cytochrome-c/protein/min for the NADPH:cytochrome-c reductase and 28 nmole NADH/mg protein/min for NADH:cytochrome b_5 reductase). Non-enzymatic lipid peroxidation, detectable in the presence of ascorbic acid (Schaefer, 1975) was not influenced by heme-nonapeptide (not shown).

It has been established that there is a transient increase in the lipid peroxide content of liver microsomes during lipid peroxidation stimulated by ADP-Fe complex (Tam and McCay, 1970). In brain microsomes the maximum of lipid peroxide concentration was observed after 15 minutes, and at this point of time a concentration dependent inhibition was found by heme-nonapeptide on lipid peroxide accumulation (Fig. 2).

Oxygen consumption of liver microsomes in the presence of

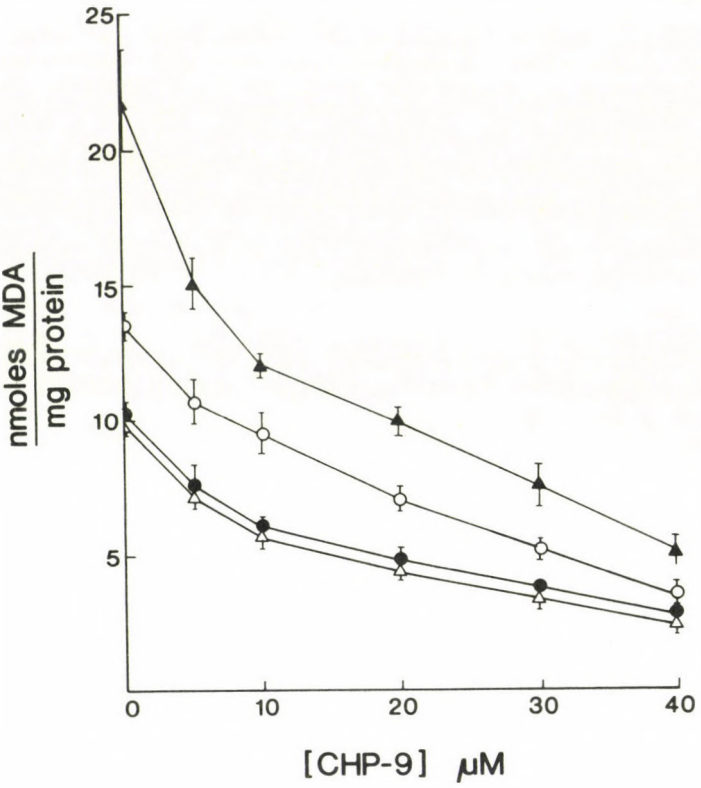


Fig. 1. Inhibition of MDA by CHP-9 in the presence of NADH or NADPH. Conditions: in 50 mM Tris-maleate buffer (pH 6.8), 300 μM NADH (Δ) or NADPH (●), and 20 μM $FeCl_3$, 2 mM ADP in the presence of NADH (▲) or NADPH (o). Reaction time was 20 min.

NADPH is markedly stimulated by heme-nonapeptide and it is nearly equivalent to NADPH oxidation (Venekei et al., 1984). As shown in Table 1, the oxygen consumption of brain microsomes increased 3-fold by heme-nonapeptide (30 μM). When ADP-Fe^{3+} was present, the stimulation was 6-fold in the presence of NADH or NADPH, either. However, this high rate was slightly increased by the addition of heme-nonapeptide.

The initiation of lipid peroxidation is considered to be due to the effect of OH radical, which is produced from the superoxide anion. In a model experiment, superoxide was generated by the xanthine oxidase - xanthine system in the presence

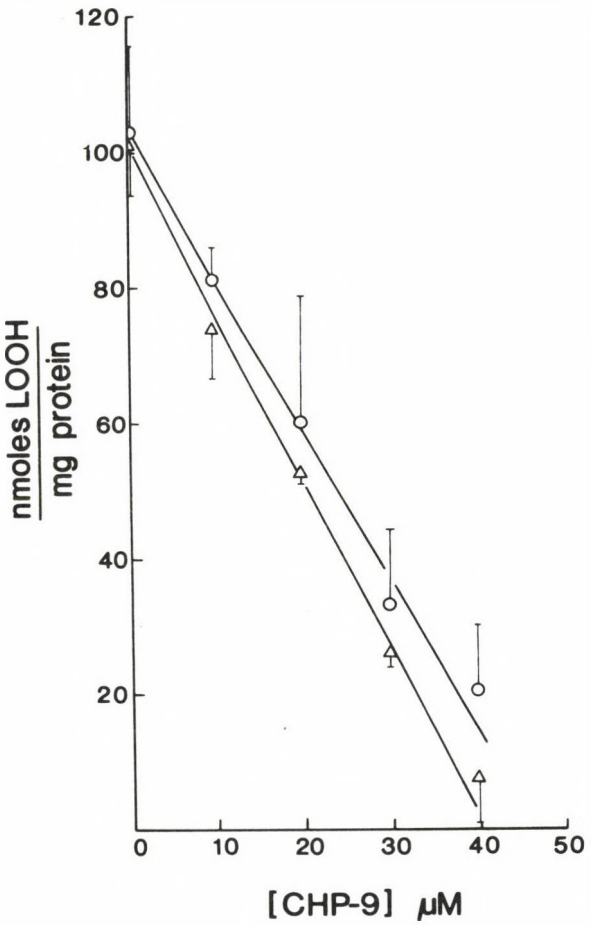


Fig. 2. Inhibition of LOOH formation by CHP-9 in the presence of NADH and NADPH. Conditions: in 50 mM Tris-maleate buffer (pH 6.8), 1 mM KH_2PO_4, 20 μM $FeCl_3$, 2 mM ADP, 300 μM NADH (Δ) or NADPH (o). Measured at the 15th min. Lipid peroxide content was 2 nmole/mg protein.

of catalase, and assayed by the reduction of cytochrome-c or tetrazolium-blue. The reduction of these indicators was inhibited by superoxide dismutase. The reduction was also inhibited by heme-nonapeptide and this inhibition was abolished by cyanide (Fig. 3).

The participation of the ADP-Fe complex in the microsomal lipid peroxidation may be connected with the reversible reduction of the iron in the complex. In order to detect the reduced

Table 1. Oxygen consumption during microsomal lipid peroxidation in the presence of NADH + H^+ and NADPH + H^+

Addition	O_2 consumption nmole O_2/mg protein/min	
	300 µM NADH	300 µM NADPH
-	5.6 ± 1.2	6.0 ± 1.5
30 µM c-Heme-nonapeptide	19.2 ± 2.0	17.6 ± 1.8
2 mM ADP-20 µM Fe^{3+}	33.6 ± 3.5	30.0 ± 3.0
ADP-Fe^{3+} + c-heme-nonapeptide	36.0 ± 4.0	32.0 ± 4.5

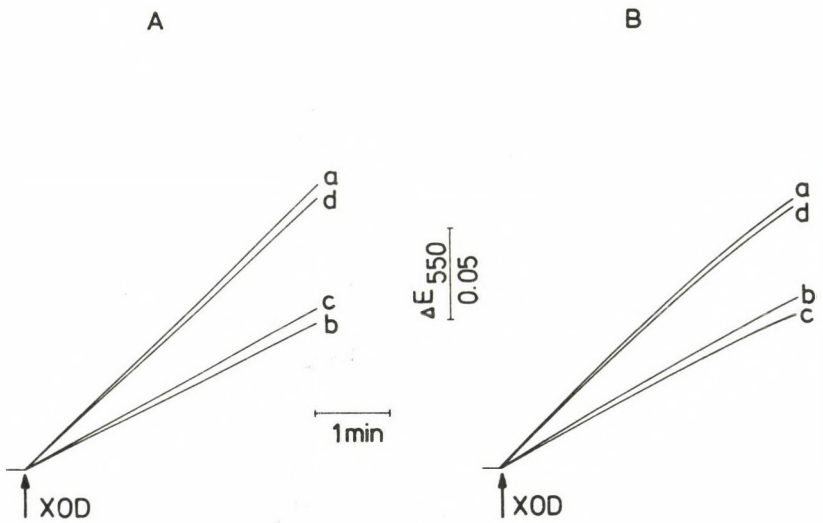


Fig. 3. Superoxide dependent reduction of cytochrome-c (part A) and nitroblue-tetrazolium (part B). Superoxide was generated by 50 µM/ml xanthine oxidase and 50 µM xanthine in the presence of 10 U/ml catalase, in 0.1 M potassium phosphate, 2 mM EDTA (pH 7.8), at 25°C. The concentration of cytochrome-c and NBT were 20 µM and 200 µM, respectively. "a": control, "b": control + 1 U/ml SOD, "c": control + 1 µM CHP-9, "d": control + 1 µM CHP-9 and 1 mM KCN. Curve "a" was the same in the presence and in the absence of KCN.

form of the complex, reaction mixtures similar to those used for the measurement of lipid peroxidation (cf. Methods) were incubated at 37°C then frozen in liquid nitrogen and analysed for $^{57}Fe^{2+}$ by Mössbauer spectrography. Published Mössbauer spectrum of ADP-Fe^{3+} was reproduced (Rabinowitz, 1966). We were unable to detect the signals characteristic for Fe^{2+} even in samples exhibiting intensive, ADP-Fe stimulated peroxidation. Considering the sensitivity of this experimental system, we concluded that the amount of Fe^{2+} was less than 4% of total iron in the samples.

DISCUSSION

As we have shown in this report heme-nonapeptide, derived from cytochrome-c, inhibited the NADPH and NADH dependent lipid peroxidation of brain microsomes in the presence and in the absence of ADP-Fe complex in a dose dependent manner (Fig. 1). In the case of brain microsomes the NADH dependent lipid peroxidation was much more intensive than in liver microsomes (Venekei et al., 1984), and the inhibition exerted by heme-nonapeptide was also more marked. Accordingly, the transient accumulation of lipid peroxides was also inhibited by heme-nonapeptide in a dose-dependent manner (Fig. 2).

The inhibitory effect of heme-nonapeptide on lipid peroxidation can be explained by the trapping of electrons coming from NADH of NADPH via the reductase enzymes, and/or by the quenching of oxy-radicals by the heme-nonapeptide. Regarding the first possibility, it has to be taken into account that heme-undecapeptide is an autooxidizable substrate for both purified NADPH:cytochrome P450 (cytochrome-c) reductase (Kramer and Horváth, 1983) and NADH:cytochrome-b_5 reductase (Végh and Horváth, 1982).

Besides, ADP-Fe stimulated lipid peroxidation of liver and brain microsomes is accompanied by intensive oxygen consumption. Since the high rate of ADP-Fe stimulated oxygen consumption was not increased significantly by the addition of heme-nonapeptide, we suppose that electrons of NADH and NADPH that necessary for lipid peroxidation, were trapped by the heme-nonapeptide.

Another possibility is that the heme-nonapeptide interferes with the formation of the OH radical, which has been considered responsible for the initiation of lipid peroxidation (Svingen et al., 1979). Bodaness (1983) and Clore et al. (1981) have found that the decomposition of H_2O_2 is promoted by heme-undecapeptide. We demonstrated in this paper that the reduction of cytochrome-c or tetrazolium-blue by superoxide anion radical (generated by xanthine oxidase) was inhibited by the heme-nonapeptide. Since superoxide is the first radical formed in the course of lipid peroxidation, and therefore it can be regarded as a precursor of the OH radical, the whole process may be blocked by the heme-nonapeptide at this stage.

The mechanism of stimulation of lipid peroxidation by ADP-Fe is not known in detail. It has been suggested that the reduced complex is responsible for the formation of oxyradicals (Svingen et al., 1979). Recent preliminary investigations (Ming et al., 1979) indicate, however, that the iron of the ADP-Fe^{3+} complex cannot be reduced by either NADPH:cytochrome P450 (cytochrome-c) reductase or NADH:cytochrome-b_5 reductase enzymes. These results were confirmed in our laboratory (Végh et al., unpublished data). Our effort to detect the reduced form of the complex by Mössbauer spectrography in a microsomal system exhibiting vigorous ADP-Fe stimulated peroxidation remained unsuccessful (cf. Results). Because of the limitations of this experimental system, the presence of small amounts of ADP-Fe^{2+} could not be excluded. Thus, the suggested role of the ADP-Fe^{3+} complex in the initiation of lipid peroxidation has still to be substantiated.

REFERENCES

Bodaness, R.S. (1983) Biochem. Biophys. Res. Commun. 113, 710

Buege, J.A., Aust, S.D. (1978) Methods Enzymol. 52, 302

Clore, M.G., Holloway, M.R., Orengo, C., Peterson, J., Wilson, M.T. (1981) Inorganica Chim. Acta 56, 143

Hochstein, P., Ndrdenbrand, K., Ernster, L. (1964) Biochem. Biophys. Res. Commun. 14, 323

Kramer, M., Horváth, I. (1983) Abstr. of the 15th FEBS Meeting Bruxelles, No. S-13 Fr 222

Lowry, O.H., Rosebrough, N.H., Farr, A.L., Randall, J.R. (1951) J. Biol. Chem. 193, 265

McCord, J.M., Fridovich, J. (1969) J. Biol. Chem. 244, 6049

Ming Tien, Morehouse, L.A., Bucher, J.R., Aust, S.D. (1979) Arch. Biochem. Biophys. 218, 450

Murata, M., Baba, Y., Matsuo, E., Yasuola, H., Okonogi, T. (1973) Yakugaku Zasshi 93, 762

Player, T.I., Horton, A.A. (1981) J. Neurochem. 37, 422

Rabinowitz, I.N. (1966) J. Am. Chem. Soc. 88, 4346

Rogers, M.J., Srittmatter, P.R. (1973) J. Biol. Chem. 284, 800

Schaefer, A. (1975) Biochem. Pharmacol. 29, 1781

Svingen, B.A., Buege, J.A., Neal, F.O., Aust, S.D. (1979) J. Biol. Chem. 254, 5892

Tam, B.K., McCay, P.B. (1970) J. Biol. Chem. 245, 2295

Venekei, I., Kittel, Á., Horváth, I. (1985) Acta Biochim. Biophys. Hung. (in press)

Végh, M., Horváth, I. (1982) Abstr. of the 22nd Meeting of the Hung. Biochem. Soc., Acta Biochim. Biophys. Acad. Sci. Hung. 17, 160

Waud, W.R., Brady, F.O., Wiley, R.D. (1975) Arch. Biochem. Biophys. 169, 695

Wermilion, J.L., Coon, M.J. (1974) Biochem. Biophys. Res. Commun. 60, 595

Younes, M., Weser, U. (1976) FEBS Letters 61, 209

Acta Biochim. Biophys. Hung. 21(1-2), pp. 13-22 (1986)

EFFECT OF A HEME-PEPTIDE DERIVED FROM CYTOCHROME-C ON LIPID PEROXIDATION. II. EXPERIMENTS WITH LIVER MICROSOMES

I. Venekei, Á. Knittel, I. Horváth

Second Institute of Biochemistry, Semmelweis University Medical School, Budapest, Hungary

(Received November 30, 1984)

SUMMARY

ADP-Fe^{2+} stimulated, NADPH dependent lipid peroxidation of liver microsomes (as measured by malondialdehyde formation) was not inhibited by c-heme-nonapeptide, unlike the same process in brain microsomes. However, in the presence of 5 mM aminopyrine (causing partial inhibition) or SKF-525A (a sepcific inhibitor of cytochrome P450) the residual activity of lipid peroxidation of liver microsomes was markedly inhibited by c-heme-nonapeptide. Further, c-heme-nonapeptide itself prevented the transient accumulation of lipid hydroperoxides during ADP-Fe^{2+} stimulated lipid peroxidation. These results led us to suggest two different pathways of lipid peroxidation. The first route involves cytochrome P450. The second pathway, which can be inhibited by c-heme-nonapeptide, appears to be more important physiologically.

INTRODUCTION

Hepatic accumulation of malondialdehyde, the end product of lipid peroxidation can be prevented by the administration of heme-peptides in pathological states, as described by Murata et al. (1975). It has been demonstrated in our laboratory that c-heme-nonapeptide, derived from cytochrome-c inhibited the lipid peroxidation of isolated brain microsomes (Vodnyánszky et al., 1986). Our initial observation on liver microsomes that the lipid peroxidation was not inhibited by c-heme-nonapeptide prompted us to examine the specific features of the hepatic oxidative processes. It is known that the activity of cyto-

Abbreviations: MDA, malondialdehyde; CHP-9, c-heme-nonapeptide

Akadémiai Kiadó, Budapest

chrome P450 dependent, mixed function mono-oxygenase system is very high in liver microsomes as compared to brain microsomes (Sasame et al., 1977; Benedetto et al., 1981). Therefore, we investigated the effect of c-heme-nonapeptide on lipid peroxidation of liver microsomes in the presence of substances which influence the activity of cytochrome P450. Our results suggested the existance of two, largely independent pathways of lipid peroxidation in the liver microsomes.

MATERIALS AND METHODS

Glucose-6-phosphate dehydrogenase (from yeast, grade II 250 U/ml) was from Boehringer (Mannheim), SKF-525A was a product of Sigma (St. Louis, MO.). C-heme-nonapeptide, prepared from horse heart cytochrome-c, glucose-6-phosphate, thiobarbituric acid and all other chemicals were obtained from Reanal (Hungary).

Liver microsomes were prepared from 20-25 g CFLP male mice. Livers of decapitated animals were cut with scissors and homogenized in 5 volume of ice cold 0.15 M potassium phosphate buffer, pH 7.5. The homogenate was centrifuged at 9 000 x g for 20 min. The supernatant was sedimented at 105 000 x g for 60 min. The pellet was suspended in 110 volumes of Tris-HCl buffer (0.1 M Tris-HCl, 50 mM KCl, pH 7.5), and was ultracentrifuged again as above. The microsomal pellet was stored, without being suspended, for maximum two weeks at -22°C. During this period the parameters characterizing lipid peroxidation (O_2 consumption, MDA formation), as well as cytochrome-c reductase and NADPH oxidase activities remained unaltered.

The incubation mixture contained 0.4 mg/ml microsomal protein in 0.1 M Tris-HCl buffer (pH 7.5) and 50 mM KCl. Incubation was performed at 30°C in a reaction mixture containing 0.3 mM NADPH plus regenerating system (5 mM glucose-6-phosphate, 0.05 U/ml glucose-6-phosphate dehydrogenase). Further additions are specified in Legends to figures and tables. After two minutes of preincubation the reaction was started by adding the appropriate amount of microsomes to the reaction mixtures.

Lipid peroxidation was followed by measuring malondialdehyde concentration using the thiobarbituric acid assay (Ottolenghi, 1959). Lipid hydroxyperoxide content of the microsomes was determined iodometrically according to Buege and Aust (1978).

O_2 consumption was measured by a Clark-type O_2 electrode. Cytochrome-c reduction was measured at 550 nm in the reaction mixture described above except that microsome concentration was 10 µg protein/ml. Their reaction was started by addition of cytochrome-c or c-heme-nonapeptide, to a final concentration of 20 µM. NADPH oxidation was measured in the same reaction mixture as used for determination of cytochrome-c reduction, but it did not contain the NADPH regenerating system. Initial concentration of NADPH in the cuvette was 100 µM. The measurements were performed in thermostated

(30°C) cuvette block of a Beckman Model 25 spectrophotometer, in 0.5 ml final volume.

Protein concentration was measured by the method of Lowry et al. (1951).

RESULTS

The effect of heme-peptides (derived from cytochrome-c) was investigated on the ADP-Fe^{2+} stimulated lipid peroxidation of liver microsomes, which is the most studied in vitro model system. As shown in Fig. 1, c-heme-nonapeptide did not influence the ADP-Fe^{2+} stimulated malondialdehyde formation (similar results were obtained with heme containing octa- and undeca peptides of cytochrome-c). In the absence of ADP-Fe^{2+}, the

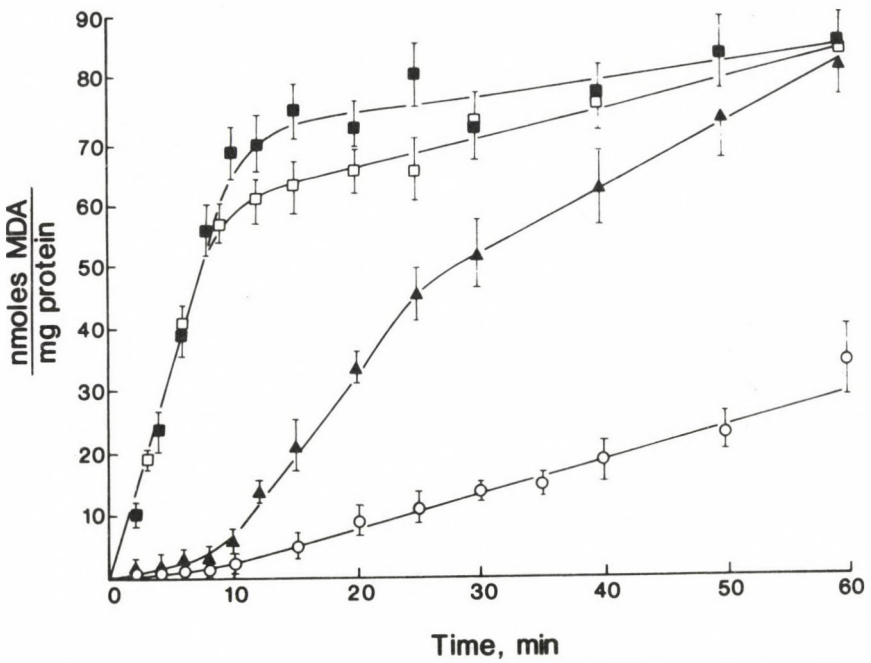


Fig. 1. Effect of c-heme-nonapeptide on the time course of ADP-Fe^{2+} stimulated MDA formation. Incubation mixture contained the following additions: 20 μM $FeSO_4$ and 2 mM ADP (■), 20 μM $FeSO_4$, 2 mM ADP and 20 μM c-heme-nonapeptide (□), 20 μM c-heme-nonapeptide (▲), no addition (o). The latter was referred as endogeneous MDA formation. For the composition of incubation mixture see Materials and methods.

c-heme-peptides caused an increase of malondialdehyde formation but only after a lag period of about 15 min (Fig. 1); citochrome c had no such effect, while c-heme itself was inhibitory (not shown). Further investigations on the enhancing effect of c-heme peptides will be published elsewhere.

The result that the c-heme-peptides did not inhibit the ADP-Fe^{2+} stimulated malondialdehyde formation was difficult to reconcile with the data obtained in vivo by Murata et al. (1975) The inhibitory effect of c-heme-nonapeptide manifested itself, however, in the presence of substances which affect cytochrome P450. Aminopyrine, a substrate for microsomal drug metabolizing system was added to the reaction mixture at a low concentration (5 mM) causing 25% inhibition of lipid peroxidation. As shown in Fig. 2, the magnitude of the inhibition of malondialdehyde for-

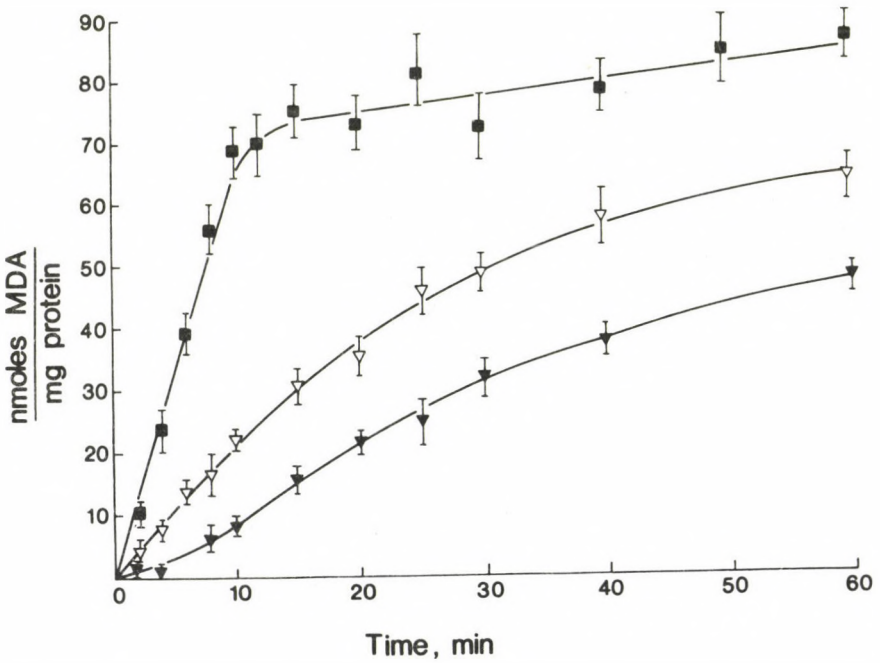


Fig. 2. Effect of c-heme-nonapeptide and aminopyrine on the time course of ADP-Fe^{2+} stimulated MDA formation. Incubation mixture contained the following additions beside 20 μM $FeSO_4$ and 2 mM ADP (■), 5 mM aminopyrine (▽), 5 mM aminopyrine and 20 μM c-heme-nonapeptide (▼). For the composition of incubation mixture see Materials and methods

mation was doubled when c-heme-peptide was also added. Further, SKF-525A (a specific inhibitor of cytochrome P450) which is known to inhibit lipid peroxidation (Miles et al., 1980; Svingen et al., 1979) was applied in combination with c-heme-nonapeptide (Fig. 3). The inhibition of malondialdehyde formation (measured at 40 min), caused by different concentrations of SKF-525A, was markedly enhanced by c-heme-nonapeptide.

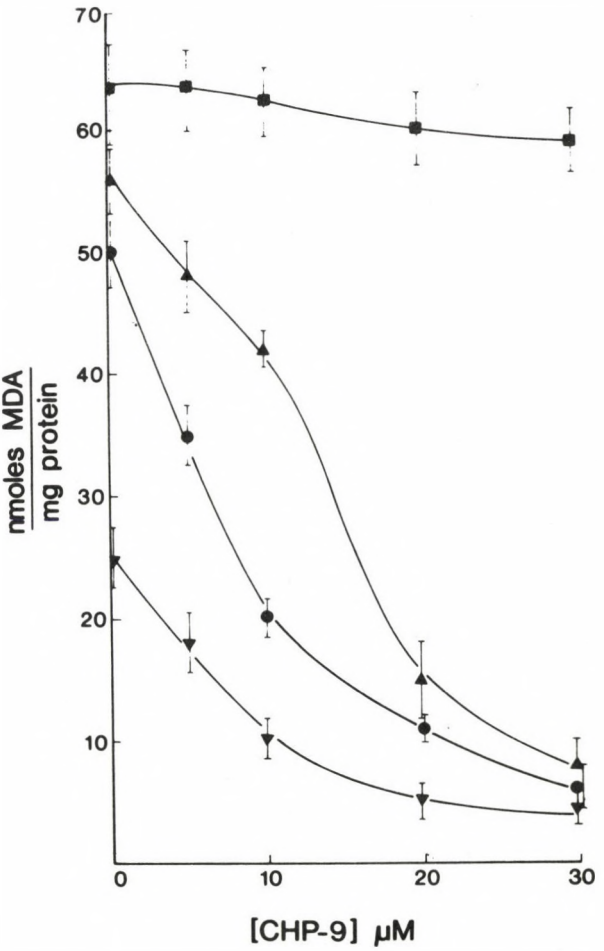


Fig. 3. Inhibitory effect of c-heme-nonapeptide and SKF-525A on MDA formation in liver microsomes. The following additions were made to incubation mixtures beside 20 μM $FeSO_4$ and 2 mM ADP: 0 μM (■), 25 μM (▲), 50 μM (●) and 100 μM (▼) SKF-525A. The MDA content was determined 40 min after starting the reaction. For the composition of incubation mixture see Materials and methods.

Liver microsomes exhibit a transient accumulation of lipid hydroperoxides during ADP-Fe^{2+} stimulated lipid peroxidation (Tam and McCay, 1970). The maximum concentration of these substances was found around the 12th minute of the incubation. The endogeneous lipid hydroperoxide content of liver microsomes was considerably higher than that of brain microsomes, as determined iodometrically, which might reflect a difference in the composition of these organelles rather than an artificial elevation during preparation.

The effect of c-heme-nonapeptide and SKF-525A on lipid hydroperoxide accumulation is summarized in Table 1. It is notable that c-heme-nonapeptide itself abolished the ADP-Fe^{2+} stimulated accumulation of hydroperoxides, while the inhibitory effect of SKF-525A was less marked. No synergism was observed between these substances. Earlier it has been established that the high initial lipid hydroperoxide content of liposomes was not decreased by c-heme-nonapeptide (not shown).

The influence of c-heme-nonapeptide on microsomal O_2 consumption and NADPH oxidation is shown in Table 2. ADP-Fe^{2+} and c-heme-nonapeptide increased the O_2 consumption but no synergism was found. NADPH oxidation was markedly increased by c-heme-nonapeptide and also by cytochrome-c. The reduced heme-compound could be detected in the case of cytochrome-c but not with c-heme-nonapeptide. Similar results were obtained previously with brain microsomes (Vodnyánszky et al., 1986).

DISCUSSION

The in vitro study of NADPH dependent, ADP-Fe^{2+} stimulated lipid peroxidation on isolated microsomes seemed a useful model system for the elucidation of the in vivo inhibitory effect of the c-heme-peptides (Murata et al., 1975). The present results obtained with liver microsomes, together with the data of the brain microsomes (Vodnyánszky et al., 1986), provided not only an in vivo verification of the interference of c-heme-nonapeptide with lipid peroxidation but they also called attention to the greater complexity of the hepatic processes.

Table 1. Lipid hyperoxide content of microsomes in endogeneous and ADP-Fe^{2+} stimulated lipid peroxidation, in the presence of c-heme-nonapeptide and SKF-525A

Lipid hyperoxide concentration was determined iodometrically in the 12th min of incubation, where lipid hyperoxide content had a maximum in ADP-Fe^{2+} stimulated lipid peroxidation (see the text). The incubation mixture contained 20 μM Fe_2SO_4 and 2 mM ADP in the case of ADP-Fe^{2+} stimulated lipid peroxidation and the following additions were made as indicated: 50 μM SKF-525A and 20 μM c-heme-nonapeptide. For the composition of incubation mixture see Materials and methods.

Addition	Lipid hyperoxide (nmol/mg protein)	
	Endogeneous	ADP-Fe^{2+} stimulated
-	117.4 ± 11.6	454.7 ± 44.6
SKF-525A	122.5 ± 18.3	325.4 ± 40.7
C-heme-nonapeptide	137.3 ± 10.7	151.2 ± 19.7
SKF-525A + c-heme-nonapeptide	100.5 ± 9.2	154.2 ± 7.0

C-heme-nonapeptide increased considerably the O_2 consumption and NADPH oxidation of liver micorosmes (Table 2) as well as of brain microsomes. C-heme-nonapeptide is a reoxidizable substrate of NADPH:cytochrome-c (cytochrome P450) reductase (Kramer and Horváth, 1983) and of NADH:cytochrome-b_5 reductase (Végh and Horváth, 1982). The increased oxidation of NADPH has been regarded as one of the possible sites of interference of c-heme-peptides with lipid peroxidation, because of the trapping of electrons. Another disturbing effect of c-heme-nonapeptide might be the quenching of superoxide radicals (Vodnyánszy et al., 1986), which are thought to be necessary for the initiation of lipid peroxidation (Kuo-Lan Fong et al., 1973; Tien et al., 1982; Willson, 1979).

Table 2. Effect of ADP-Fe^{2+} and C-heme-nonapeptide on the O_2 consumption, NADPH oxidation and c-heme- reduction of microsomes

The following additions were made to incubation mixtures as indicated: 20 μM Fe_2SO_4, 2 mM ADP, 20 μM c-heme-nonapeptide and 20 μM cytochrome-c. The composition of incubation mixtures and assays used are described under Materials and methods. Measurements were performed in the first five minutes after addition of microsomes.

Addition	O_2 consumption	NADPH oxidation	c-heme reduction
	nmol/mg protein/min		
-	18.8 ± 4.7	0.0	-
ADP-Fe^{2+}	70.2 ± 15.8	6.2 ± 2.9	-
C-heme-nonapeptide	73.1 ± 10.7	119.3 ± 11.9	0.0
ADP-Fe^{2+} + c-heme-nonapeptide	76.9 ± 13.5	116.7 ± 10.8	0.0
Cytochrome-c	22.7 ± 5.2	66.7 ± 8.6	80.3 ± 14.8

The peculiar feature of the lipid peroxidation of liver microsomes was the following. In the presence of ADP-Fe^{2+}, the transient accumulation of lipid hydroperoxides (measured iodometrically) was much more inhibited (90%) by 20 μM c-heme-nonapeptide than by SKF-525A (Table 1), whereas the formation of malondialdehyde (end product of lipid peroxidation) was hardly affected by c-heme-nonapeptide but strongly decreased by SKF-525A at the same concentrations (Fig. 3). In the case of brain microsomes, however, c-heme-nonapeptide had a clear-cut, concentration dependent inhibitory effect on ADP-Fe^{2+} stimulated malondialdehyde formation (Vodnyánszky et al., 1984). This striking difference between the sensitivity of malondialdehyde formation in liver and brain microsomes towards the inhibitory effect of c-heme-nonapeptide was traced back to the involvement

of cytochrome P450 which is much more abundant in liver microsomes (Sasame et al., 1977; Benedetto et al., 1981). Blocking of the function of cytochrome P450 by a specific inhibitor, SKF-525A (Fig. 3) or the charging of the drug metabolism system (of which cytochrome P450 is an important member) by a substrate, aminopyrine (Fig. 2) resulted in the "restoration" of the c-heme-peptide inhibition of malondialdehyde formation in liver microsomes.

The participation of cytochrome P450 in the propagation of lipid peroxidation has been suggested earlier (Svingen et al., 1979). Our in vitro observations indicate that, beside the c-heme-peptide sensitive mechanism (dominating in brain microsomes), a shunt of lipid peroxidation involving cytochrome P450 is present in liver microsomes. This shunt could be responsible for the intensive malondialdehyde formation in the presence of c-heme-nonapeptide (Figs. 1 and 3). Malondialdehyde formation by this route was apparently independent of lipid hydroperoxide accumulation, and cytochrome P450 might be responsible for the initiation and/or multiplication phases of lipid peroxidation. However, the function of this shunt in vivo may be blocked by endogenous substrates of the drug metabolizing (cytochrome P450) system, therefore the lipid peroxidation occures via c-heme-peptide sensitive route, which is in agreement with the results of Murata et al. (1975).

REFERENCES

Benedetto, C., Dianzani, M.V., Cheeseman, A.M. (1981) Biochim. Biophys. Acta 677, 363

Buege, J.A., Aust, S.D. (1978) Methods Enzymol. 52, 302

Kramer, M., Horváth, I. (1983) Abstract of the 15th FEBS Meeting, Bruxelles, No. S-13, Fr-222

Kuo-Lan Fong, McCay, P.B., Poyer, J.L. (1973) J. Biol. Chem. 248, 7792

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193, 265

Miles, P.R., Wright, J.R., Bowman, L., Colby, H.D- (1980) Biochem. Pharmacol. 29, 565

Murata, M., Masahiro, M., Yasuda, H., Hiroshi, Y., Baba, Y., Yoshihiko, B. (1975) Yakugaku Zasshi 95, 659

Ottolenghi, A. (1959) Arch. Biochem. Biophys. 79, 355

Saseme, H.A., Ames, M.M., Nelson, S.D. (1977) Biochem. Biophys. Res. Commun. 78, 919

Svingen, B.A., Buege, J.A., O'Neal, F.O., Aust, S.D. (1979) J. Biol. Chem. 254, 5892

Tam, B.K., McCay, P.B. (1970) J. Biol. Chem. 245, 2295

Tien, M., Svingen, B.A., Aust, S.D. (1982) Arch. Biochem. Biophys. 216, 142

Végh, M., Horváth, I. (1982) Acta Biochim. Biophys. Acad. Sci. Hung. 17, 160

Vodnyánszky, L., Marton, A., Végh, M., Blázovits, A., Auth, F., Vértes, A., Horváth, I. (1986) Acta Biochim. Biophys. Hung. 21, 3

Acta Biochim. Biophys. Hung. 21(1-2), pp. 23-31 (1986)

NEUROTRANSMITTER UPTAKE BY THE CENTRAL NERVOUS SYSTEM OF FRESH-WATER MUSSEL (ANODONTA CYGNEA L): I. IN VITRO UPTAKE OF SEROTONIN, DOPAMINE AND NORADRENALINE

J. Nemcsók, Z. Rakonczay[§]

Biological Research Institute, Hungarian Academy of Sciences, Tihany, and [§]Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

(Received August 28, 1984)

SUMMARY

The accumulation of serotonin, dopamine and noradrenaline in the cerebral, pedal and visceral ganglia is mediated by a high-affinity (uptake$_1$) and a low-affinity (uptake$_2$) system. This uptake mechanism is dependent on time and temperature.

The monoamine uptake systems in the cerabral, pedal and visceral ganglia are not identical. At low external serotonin concentration, the cerebral ganglia display twice the rate of uptake$_1$ as compared to the visceral and pedal ganglia.

INTRODUCTION

Amine reuptake as a possible mechanism for the inactivation of synaptically released monoamines has been extensively studied in vertebrates (Iversen, 1967). Some of the earlier studies (Robinson et al., 1965) described the accumulation of serotonin (5HT) by brain slices and by subcellular particles as being a temperature - dependent process. However, after careful analysis of this uptake by these authors (Iversen, 1967; Robinson et al., 1965) concluded that were merely examining an ion-exchange process. More recently, by using low concentrations of 5HT, several workers, obtained evidence for the energy-requiring,

Present adress: Department of Biochemistry, József Attila University, [§]Szeged, Hungary
Central Research Laboratory, Medical University, Szeged, Hungary

Akadémiai Kiadó, Budapest

specific uptake of 5HT by brain slices. Aghajanian and Bloom (1967) found convincing evidence in an in vivo system that exogenous triatiated 5HT labelled serotoninergic neurons specifically. However, other in vivo studies shed doubt on the specificity of exogenous 5HT accumulation by serotoninergic neurons. Lichtensteiger et al. (1967) administered large doses of 5HT intraperitoneally and examined the brain by histochemical fluorescence techniques. A considerable amount of the 5HT administered was accumulated by dopaminergic neurons in the brain.

Using the Michaelis-Menten equation Shaskan and Snyder (1970) calculated that low concentrations (maximum 10^{-6} M) of exogenous 5HT labelled 5HT neurons specifically, but concentrations above 10^{-6} M were also accumulated by dopaminergic neurons.

Uptake in invertebrates has received relatively little attention and only recently has biochemical evidence indicated that uptake in molluscs may have pharmacological and kinetic properties similar to those of mammalian uptake systems (Carpenter et al., 1971; Myers and Sweeney, 1973).

Earlier data (Salánki et al., 1974) supported the transmitter role of 5HT, dopamine (DA) and noradrenaline (NA) at the interneuronal level in the CNS of Anodonta. We have recently studied the accumulation of these amines from a kinetic point of wiew, not only in the pedal ganglia (Hiripi et al., 1975), but also in the cerebral and visceral ganglia. The latter ganglia play an important role in the regulation of periodic activity.

Experiments were also designed to determine how the three types of Anodonta ganglia accumulate 5HT, DA and NA quantitatively and qualitatively.

MATERIALS AND METHODS

In our experiments, specimens of the fresh-water mussel (Anodonta cygnea L.) weighing 200-300 g were used. After dis-

Abbreviations: DA, dopamine; NA, noradrenaline; CA, catecholamine; 5HT, 5-hydroxy-tryptamine (serotonin)

section, the ganglia were stored in ice-cold physiological saline (Marczynski, 1959). About 10 mg each of the cerebral, pedal and visceral ganglia from two animals was preincubated for 10 minutes at 20°C in 2 ml physiological saline solution, followed by a 30-minute incubation with 0.05-100 μM ^{3}H - labelled biogenic amines.

Az the end of the incubation period the ganglia were washed in 20 ml ice-cold physiological solution, blotted dry on filter paper, weighed and put into tubes containing 0.5 ml NCS (Nuclear Chicago Solubilizer) tissue solubilizer. Dissolved ganglia and an aliquot from the incubation medium were diluted in 10 ml Bray's solution and counted in a Nuclear Chicago liquid scintillation spectrometer. The tissue to medium ratios (R = dpm of ^{3}H per g tissue divided by counts of ^{3}H per 1 ml medium), and the rates of uptake were calculated by the method of Shaskan and Snyder (1970). To evaluate the kinetics of amine accumulation, the reciprocals of the amine accumulation rates calculated from R and the amine concentration were analyzed by Lineweaver-Burk plots.

RESULTS

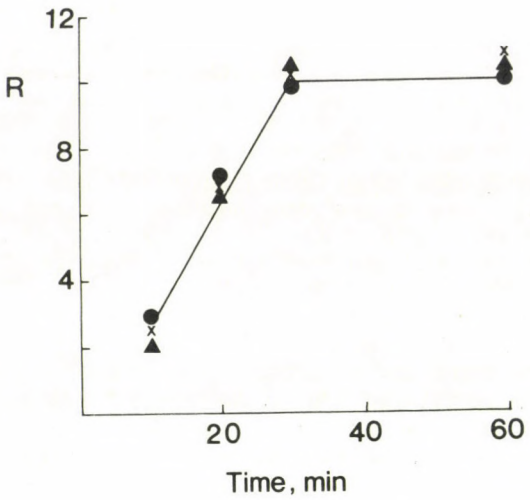
For all amines, the experimental results gave plots that could not be described by single straight lines, but could be resolved into two straight lines. This demonstrated that the accumulation process has two components with different affinities.

Uptake of ^{3}H-5HT

The rates of ^{3}H-5HT accumulation in the cerebral, pedal and visceral ganglia are illustrated in Fig. 1. Between 0 and 30 min there was a rapid linear uptake of ^{3}H-5HT. The uptake of ^{3}H-5HT was linearly dependent upon temperature between 0 and 20°C, but when the temperature was further increased to 30°C, the uptake was not greater than it was at 20°C (Fig. 2). With 5HT the Lineweaver-Burk plot could be resolved into two components: a higher-affinity component (i.e. Uptake$_1$), with K_m and V_{max} values denoted as K_{m_1} and V_{max_1}, and lower-affinity component (i.e. uptake$_2$, Fig. 3), with values K_{m_2} and V_{max_2} (Table 1).

The Michaelis-Menten equation was used to calculate the relative contributions of uptake$_1$ and uptake$_2$ to the total accumulation of the amines at a variety of amine concentrations.

At low 5HT concentrations (between 2 x 10^{-8} and 10^{-6} M) uptake$_1$ had 2-3 times the rate uf uptake$_2$ for all three ganglia



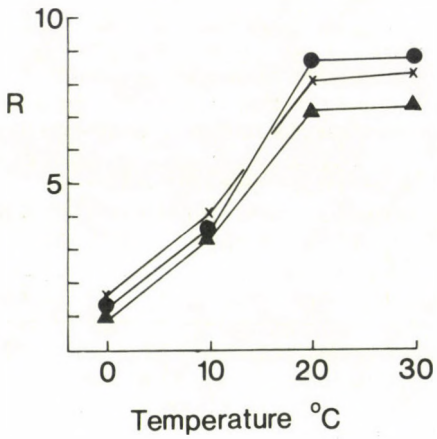


Fig. 1. (left) Rates of ^{3}H-5HT uptake by Anodonta ganglia incubated with 0.1 nmol ^{3}H-5HT at 20°C. Each point is the mean of 3 values
●——● cerabral ganglia
▲——▲ visceral ganglia
X——X pedal ganglia
The tissue to medium ratio (R) was calculated as dpm of ^{3}H in 1 g tissue per counts of ^{3}H in 1 ml medium.

Fig. 2. (right) Effect of temperature on uptake of ^{3}H-5HT by cerebral, pedal and visceral ganglia incubated with 0.1 nmol ^{3}H-5HT for 30 minutes. Symbols as in Fig. 1.

(Figs. 4 and 6). At concentrations between 5 x 10^{-6} and 10^{-6} M, the rates of uptake$_1$ and uptake$_2$ were about the same, while an additional increase in the concentration brought about a higher for uptake$_2$ and rate of uptake$_1$ approximated to the limiting value. At a concentration of 10^{-4} M for pedal and visceral ganglia, the uptake$_2$ system had a rate 40 times greater than that of uptake$_1$ (Figs. 5 and 6). At low seretonin concentrations (uptake$_1$), the cerebral ganglia accumulate twice as much serotonin as did the pedal and visceral ganglia, and at high serotonin concentrations the accumulation by uptake$_2$ was lower than that for the pedal and visceral ganglia (Fig. 4).

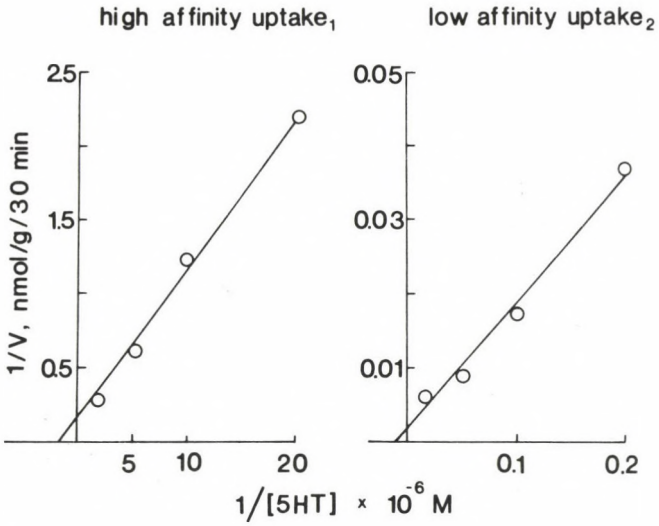


Fig. 3. Lineweaver-Burk plot of serotonin accumulation in cerebral ganglia versus 5HT concentration.

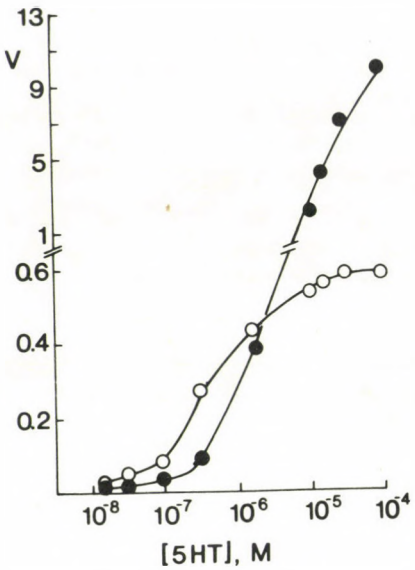
Uptakes of ^{3}H-DA and ^{3}H-NA

The uptakes of both investigated catacholamines (CA) were linear between 0 and 40 min, and dependent upon the temperature, similary to that of serotonin. At low DA concentration the K_{m_1} and V_{max_1} values, and at high DA concentration the K_{m_2} and V_{max_2} values, were about the same for cerebral, pedal and visceral ganglia (Table 1).

Uptake$_1$ by cerebral ganglia at low DA concentration had a rate twice that of uptake$_2$, but above 10^{-6} M DA uptake$_2$ had a rate 5 times greater than uptake$_1$. Uptake$_1$ for pedal ganglia predominated up to a concentration of 7.5 x 10^{-5} M DA, whereas above this concentration uptake$_2$ had a much greater rate than uptake$_1$. Uptake$_1$ for visceral ganglia predominated over uptake$_2$ up to a concentration of 10^{-7} M DA only.

Above 10^{-6} M DA, uptake$_2$ had a rate 15-20 times higher than uptake$_1$ for all three types of ganglia.

In the case of NA, uptake$_1$ predominated over uptake$_2$ up to a concentration of 10^{-6} M for all three ganglia. Above



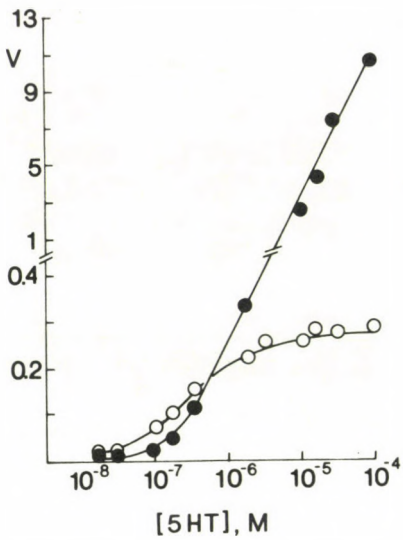


Fig. 4. (left) Rates of 5HT accumulation in Anodonta cerebral ganglia via $uptake_1$ (o) and $uptake_2$ (•) at various 5HT concentrations at 20°C. Kinetic constants for the two 5HT uptake were determined by the Michaelis-Menten equation.

Fig. 5. (right) Rates of 5HT accumulation in Anodonta pedal ganglia via $uptake_1$ (o) and $uptake_2$ (•) at various 5HT concentrations at 20°C.

10^{-6} M, NA accumulated in all three ganglia mainly by $uptake_2$. The K_{m_1}, V_{max_1}, K_{m_2} and V_{max_2} values were about the same for all three ganglia (Table 1)

DISCUSSION

The present study has shown that similarities exist between the monoamine uptake mechanisms of invertebrates and vertebrates (Shaskan and Snyder, 1970; Snyder and Coyle, 1969; Iversen, 1970; 1971) and snails (Osborne et al., 1975). The cerebral, pedal and visceral ganglia of Anodonta are able to accumulate serotonin by a high-affinity ($uptake_1$) and a low-affinity ($uptake_2$) transport system. Previous reports demonstrated that gastropod nervous tissue has the ability to accumulate 5HT (Osborne and Neuhoff,

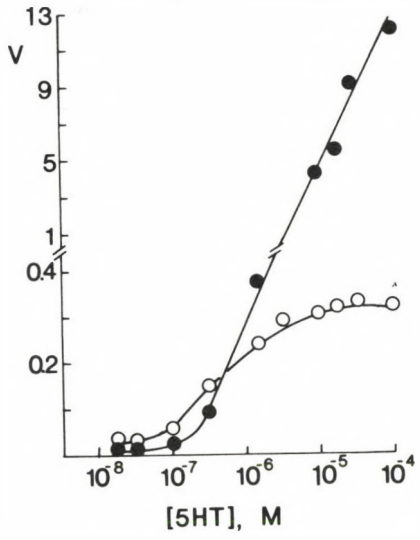


Fig. 6. Rates of 5HT accumulation in Anodonta visceral ganglia via $uptake_1$ (o) and $uptake_2$ (●) at various 5HT concentrations at 20°C.

1974; Carpenter et al., 1971; Osborne et al., 1975). Moreover, autoradiography studies have shown that nervous elements in snail (Pentreath and Cottrell, 1972; 1973) and fresh-water mussel ganglia (Elekes, 1975) are the sites which take up radioactive serotonin. At low serotonin concentrations, $uptake_1$ had twice the rate of $uptake_2$ for all three ganglia. This probably represents the uptake of serotonin only into the serotonin-storing vesicles. At high external 5HT concentration, serotonin is probably taken up by the $uptake_2$ mechanism not only into the serotonin-storing vesicles, but also into the catecholamine-storing ones into other nervous elements.

$Uptake_1$ system of cerebral ganglia had twice the rate of the others; this could be explained by the key role of cerebral ganglia in the regulation of periodic activity (Salánki, 1968), because the active serotonin pool present in cerebral ganglia could presumably be mobilized very rapidly by the effective and high-affinity $uptake_1$ system. The K_m and V_{max} values for pedal

Table 1. Kinetic constants for the rates of accumulation of ^{3}H-5HT, ^{3}H-DA and ^{3}H-NA in the cerebral, pedal and visceral ganglia

	Ganglia	High-affinity system uptake$_1$		Low-affinity system uptake$_2$	
		K_{m_1}, app. M x 10^6	V_{max_1} mol/g/min	K_{m_2} M x 10^5	V_{max_2} nmol/g/min
^{3}H-5HT	cerebral	1.11	0.33	1.25	2.22
	pedal	0.74	0.32	20.00	37.04
	visceral	0.70	0.30	16.60	31.70
^{3}H-DA	cerebral	1.43	0.61	10.00	6.66
	pedal	2.00	0.66	10.00	11.11
	visceral	0.91	0.39	5.00	16.60
^{3}H-NA	cerebral	0.56	0.23	2.86	4.44
	pedal	0.50	0.20	10.00	6.66
	visceral	0.50	0.16	2.94	2.56

ganglia are different from those reported in our earlier paper (Hiripi et al., 1975). This difference might be due to a seasonal change in uptake$_1$, as well as to alterations in the 5HT content of the CNS of Anodonta in different seasons of the year (Salánki et al., 1974). Seasonal monoamine varioation has also been demonstrated for another mussel, Mytilus edulis (Stefano and Catapane, 1977). As regards the uptakes of DA and NA, there are only slight differences in K_m and V_{max} values between the three different types of ganglia.

Unlike the case of 5HT, where at low 5HT concentration the rate of uptake$_1$ for the cerebral ganglia was twice that

for the pedal and visceral ganglia, with DA and NA uptake$_1$ is quantitatively similar in all three types of ganglia. It seems that the reuptake of DA and NA does not play a very important role in the regulation of periodic activity.

REFERENCES

Aghajanian, O., Bloom, F. (1967) J. Pharm. Exp. Ther. 156, 23-30

Carpenter, D., Breese, S., Schanberg, Kopin, I. (1971) Intern. J. Neuroscience 2, 49-56

Elekes, K. (1975) Acta Biol. Acad. Sci. Hung. 26, 225-228

Hiripi, L., Rakonczay, Z., Nemcsók, J. (1975) Annal. Biol. Tihany 42, 21-28

Iversen, L.L. (1967) The Uptake and Storoge of Noradrenaline in Sympathetic Nerves, Cambridge Univ. Press, London, New York

Iversen, L.L. (1970) Adv. in Biochemical Parmacology 2, 109-132

Iversen, L.L. (1971) Brit. J. Pharmacol. 41, 571-591

Lichtensteiger, W., Mutzner, V., Langemann, H. (1967) J. Neurochem. 14, 389-397

Myers, P.R., Sweeney, D.C. (1973) Comp. Gen. Pharm. 4, 321-325

Marczinsky, T. (1959) Bull. Acad. Pol. Sci. 7, 147-150

Osborne, N.N., Neuhoff, V. (1974) J. Neurochem. 22, 363-371

Osborne, N.N., Hiripi, L., Neuhoff, V. (1975) Biochem. Pharmacol. 24, 2141-2148

Pentreath, V.W., Contrell, G.A. (1972) Nature New Biology 239, 213-214

Pentreath, V.W., Contrell, G.A. (1973) Z. Zellforsch. 143, 21-35

Robinson, J.D. (1965) J. Pharm. Exp. Ther. 147, 236-243

Salánki, J. (1968) in Symposium on Neurobiology of Invertebrates, pp. 493-501, Akadémiai Kiadó, Budapest and Plenum New York

Salánki, J., Hiripi, L., Nemcsók, J. (1974) J. Interdiscipl. Cycle Res. 5, 227-285

Shaskan, E.G., Snyder, S.H. (1970) J. Pharm. Exp. Ther. 175, 404-418

Snyder, S.H., Coyle, J.T. (1969) J. Pharm. Exp. Ther. 165, 78-86

Stefano, G.B., Catapane, E.J. (1977) Experientia 33, 1341-1342

Acta Biochim. Biophys. Hung. 21(1-2), pp. 33-41 (1986)

NEUROTRANSMITTER UPTAKE BY THE CENTRAL NERVOUS SYSTEM OF FRESH-WATER MUSSEL (ANODONTA CYGNEA L): II. EFFECTS OF VARIOUS DRUGS, AMINES AND IONS

Z. Rakonczay, J. Nemcsók[§]

Institute of Biochemistry, Biological REsearch Center, Hungarian Academy of Sciences, Szeged, and [§]Biological Research Institute, Hungarian Academy of Sciences, Tihany, Hungary

(Received August 28, 1984)

SUMMARY

The high-affinity uptakes of serotonin, dopamine and noradrenaline by the cerebral, pedal and visceral ganglia of fresh-water mussel were inhibited by the presence of other amines.

Reserpine was the most potent inhibitor among the drugs tested. Chlorpromazine seemed to be a specific inhibitor of serotonin uptake. It did not affect the accumulation of dopamine and noradrenaline. Ouabain had an inhibitory effect only at relatively high concentrations.

Lack of sodium greatly diminished the rates of accumulation of serotonin and dopamine, but had less effect on the uptake of noradrenaline.

INTRODUCTION

Serotonin (5HT), dopamine (DA) and noradrenaline (NA) have been demonstrated in the central nervous system of mussels, and the available data suggest that these amines have transmitter functions (Gerschenfeld, 1973).

A small amount of monoamine oxidase is present in the ganglia of Anodonta (Hiripi, Salánki, 1971). Another type of inactivation, which has been proved for several transmitters, is a reuptake mechanism (Iversen, 1970; 1971). We recently

Present adress: Central Research Laboratory, Medical University, Szeged, Hungary
[§]Department of Biochemistry, József Attila University, Szeged, Hungary

Akadémiai Kiadó, Budapest

demonstrated that biogenic amines are taken up by an active transport process in Anodonta, suggesting that reuptake of 5HT, DA and NA may be a mechanism for inactivating the amines (Hiripi et al., 1975; Nemcsók, Rakonczay, 1978). The uptake is mediated by two systems, designated high-affinity uptake (uptake$_1$) and low-affinity uptake (uptake$_2$).

A specific accumulation was also revealed by autoradiography at low serotonin concentrations in Anodonta (Elekes, 1976).

The aim of the present work was to study the effects of various drugs, amines and ions on the high-affinity uptakes of serotonin, dopamine and noradrenaline by the nervous tissue of Anodonta.

MATERIALS AND METHODS

Fresh-water mussels (Anodonta cygnea L.) weighing 200-300 g were used. Cerebral, visceral and pedal ganglia were dissected and stored in ice-cold physiological saline (Marczynski, 1959). About 5-10 mg wet weight ganglia was preincubated for 5 min in the presence or absence of amines and various drugs at 25°C in 1 ml physiological saline solution. In the case of ion-free incubation medium, the ion in question was substituted by sucros to give the same osmotic pressure as for the control. Incubation was carried out at 25°C for 30 min in the presence of 0.1 µM labelled amines. The accumulation of amines at 0°C was much lowe than that at 25°C. All results presented here are without correc tion. The uptake of ^{3}H- or ^{14}C-labelled amines was linear for at least 40 min, as reported earlier (Hiripi et al., 1975). At the end of the incubation the ganglia were recovered with forcep and washed in 20 ml ice-cold physiological solutions. Preliminar experiments had established that there was no significant releas of radioactivity from the tissues during the washing process. Th ganglia were then placed in vials containing 0.5 ml NCS tissue solubilizer for at least 2 hr at room temperature, before the addition of 10 ml Bray's solution. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. The tissue to medium ratios (R = dpm of ^{3}H per g tissue divided by counts of ^{3}H or ^{14}C per 1 ml medium) were calculated by the method of Shaskan and Snyder (1970).

Quenching was corrected by the sample chanel ratio method.

The metabolism of amines during the uptake studies was checked in accordance with Osborne et al. (1975). Under the incubation conditions employed, 90% or more of the labelled amines was not metabolized.

Abbreviations: DA, dopamine; NA, noradrenaline; 5HT, 5-hydroxy-tryptamine (serotonin); CPZ, chloropromazine

The radioactive chemicals used were as follows: ethylamine-1-^{14}C dopamine hydrochloride, 59 mCi/mmol, Amersham; 5-hydroxy 6-^{3}H tryptamine creatinine sulphate, 500 mCi/mmol, Amersham; DL-7-^{3}H noradrenaline hydrochloride 11.6 Ci/mmol, Amersham. All other chemicals were of analytical grade.

RESULTS

Effects of amines on amine uptakes

The effects of amines on the uptakes of ^{3}H-5HT ^{14}C-DA and ^{3}H-NA are given in Table 1. From the IC 50 values determined, it is clear that uptakes$_1$ of 5HT, DA and NA are inhibited by the presence of other amines in the incubation medium. Inhibition of 5HT uptake by DA was about 10 times higher than that by NA. NA and DA were 10 times less potent inhibitors of 5HT uptake in cerebral ganglia than in pedal and visceral ganglia. 5HT was 7-8 times more potent in inhibiting DA uptake in cerebral and visceral ganglia than in pedal ganglia. Generally, the IC 50 values were about 10 times lower for cerebral and visceral ganglia than pedal ganglia.

Effects of pharmacological agents on amine uptakes

The effects of pharmacological agents on the uptakes of ^{3}H-5HT, ^{14}C-DA and ^{3}H-NA are listed in Table 2. Reserpine was the most potent inhibitor of the accumulation of the biogenic amines in all three ganglia. The potency sequence for inhibiting 5HT accumulation was reserpine > CPZ > imipramine > ouabain. CPZ selectively inhibited the uptake of 5HT, and did not effect the accumulation of DA and NA. Ouabain, an inhibitor of Na^{+}-K^{+} ATPase inhibited amine uptake if applied at higher concentrations.

Effects of cations in the incubation medium on amine uptakes

The effects of cations in the incubation medium on the accumulation of ^{3}H-5HT, ^{14}C-DA and ^{3}H-NA are shown in Table 3. The accumulation of 5HT, DA and NA in the ganglia requires Na^{+} ions, a lack of Na^{+} ions markedly decreased the rates of accumulation. The omission of Ca^{2+} and K^{+} ions diminished the uptakes of the amines, but this effect was not so great as in the case of Na^{+} ions. A lack of Mg^{2+} increased the accumulation of 5HT in cerebral and pedal ganglia, but the accumu-

Table 1. Effects of amines on the uptake of ^{3}H-5HT, ^{14}C-DA and ^{3}H-NA by pedal, cerebral and visceral ganglia of Anodonta

IC 50 = concentration of inhibitor (in M) required to produce 50 per cent inhibition of the uptake of ^{3}H-5HT, ^{14}C-DA or ^{3}H-NA. Each amine was tested at three concentrations, and a mean value was obtained for three determinations at each concentration. The tissues were preincubated for 5 min in the presence of amine before labelled biogenic amine was added. The IC 50 values were obtained by a graphical method. CG = cerebral ganglia; PG = pedal ganglia; VG = visceral ganglia

	CG	PG	VG
a.) ^{3}H-5HT uptake (IC 50 values)			
DA	7.0×10^{-6}	2.5×10^{-5}	10^{-5}
NA	6.5×10^{-5}	2.5×10^{-4}	4.0×10^{-4}
b.) ^{14}C-DA uptake (IC 50 values)			
5HT	9.0×10^{-6}	6.0×10^{-5}	9.0×10^{-6}
c.) ^{3}H-NA uptake (IC 50 values)			
5HT	9.5×10^{-6}	3.0×10^{-5}	4.5×10^{-6}

lation of 5HT in cerebral and pedal ganglia, but the accumulation of DA and NA decreased with the exception DA uptake by visceral ganglia.

Table 2. Effects of pharmacological agents on the uptake of ^{3}H-5HT, of ^{14}C-DA and ^{3}H-NA by pedal, cerebral and visceral ganglia of Anodonta

Each drug was tested at three concentrations. The IC 50 values were obtained by a graphical method and given in M. The tissues were preincubated for 5 min in the presence of the drug before labelled biogenic amine was added. Abbreviations see in Table 1.

	CG	PG	VG
a.) ^{3}H-5HT uptake (IC 50 values)			
ouabain	3.3×10^{-4}	1.5×10^{-4}	4.5×10^{-4}
reserpine	6.5×10^{-8}	5.5×10^{-8}	2.8×10^{-8}
imipramine	1.5×10^{-6}	8.0×10^{-6}	8.0×10^{-6}
CPZ	1.0×10^{-8}	4.0×10^{-7}	1.0×10^{-7}
b.) ^{14}C-DA uptake (IC 50 values)			
oubain	1.0×10^{-5}	2.5×10^{-4}	9.0×10^{-5}
reserpine	2.5×10^{-8}	2.0×10^{-7}	8.0×10^{-8}
CPZ*	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
c.) ^{3}H-NA uptake (IC 50 values)			
ouabain	3.0×10^{-5}	2.5×10^{-4}	4.0×10^{-4}
reserpine	3.0×10^{-8}	4.0×10^{-7}	6.5×10^{-7}
CPZ**	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}

* at this concentration the uptake of DA decreased by 10-30% as compared to the control

** at this concentration the uptake of NA decreased by 25-40% as compared to the control

Table 3. Effects of alteration of the incubation medium on the uptake of ^{3}H-5HT, ^{14}C-DA and ^{3}H-NA by pedal, cerebral and visceral ganglia of Anodonta

The results are mean values of at least 3 experiments and given as a percentage of the control. Sucrose was substituted for ions omitted from the incubation medium. Abbreviations see in Table 1.

	CG	PG	VG
a.) ^{3}H-5HT uptake			
Na^{+}-free	66.2	30.1	30.2
K^{+}-free	64.9	61.7	54.4
Ca^{2+}-free	104.6	74.0	68.5
Mg^{2+}-free	145.4	122.8	92.3
b.) ^{14}C-DA uptake			
Na^{+}-free	29.7	21.1	13.5
K^{+}-free	63.0	66.8	74.6
Ca^{2+}-free	44.6	68.1	89.9
Mg^{2+}-free	65.1	74.2	106.2
c.) ^{3}H-NA uptake			
Na^{+}-free	71.4	72.8	71.3
K^{+}-free	53.9	36.4	89.3
Ca^{2+}-free	58.0	68.9	60.9
Mg^{2+}-free	63.4	40.2	76.5

DISCUSSION

The present results demonstrate that biogenic amines, drugs and ions have large effects on the accumulation of 5HT, DA and NA. The high-affinity uptakes of 5HT, DA and NA are inhibited by the presence of other amines in the incubation medium. This means that 5HT may enter catecholaminergic neurons and vice versa, similary as for brain slices (Shaskan and Snyder, 1970). From the IC 50 values it can be seen that cerebral and visceral ganglia play an important role in the regulation of active and rest periods of mussel (Salánki, 1968).

CPZ was found to inhibit selectively the uptake of 5HT. It did not affect the uptakes of DA and NA, in contrast to the case of squid brain, where CPZ was shown to inhibit the uptakes of both NA and 5HT (Pollard et al., 1975). CPZ might act by competing with 5HT for specific receptor sites, or it may block the actual permeation mechanism. The most potent inhibitor was reserpine, which was found to be equally effective in inhibiting the uptakes of 5HT, DA and NA similarly to the results obtained with vertebrate tissue (Iversen, 1973). In contrast, in the suboesophageal ganglia and auricle of the snail (Osborne et al., 1975; Hiripi et al., 1976) reserpine did not inhibit the accumulation of 5HT and DA.

Ouabain inhibited the accumulation of 5HT, DA and NA, suggesting a Na^+-K^+ ATPase amine transport system similar to that found in vertebrates (Bonting et al., 1961; Gorman and Marmor, 1974).

Variation of the content of the medium greatly influenced the uptakes of the biogenic amines. In many systems a Na^+ gradient is generally assumed to be one of the sources of energy for uptake against a concentration gradient. Therefore, it is not surprising that the high-affinity transport systems showed a dependence on Na^+. Sodium ions are essential for uptake of amines, and a sodium-free medium greatly decreased the accumulation of the biogenic amines.

The lack of Mg^{2+} ions increased the accumulation of 5HT. Magnesium can therefore be considered a modulationg factor of

the accumulation process.

The absence of Ca^{2+} ions from the incubation medium diminished the uptakes of DA, NA and 5HT by all types of ganglia, except for the 5HT uptake by cerebral ganglia.

Elimination of K^{+} from the incubation medium inhibited the uptakes of the biogenic amines, though the accumulation of NA in the cerebral and pedal ganglia was the most affected.

Taken as a whole, the present results demonstrate that monovalent and bivalent cations may alter the uptakes of biogenic amines and in this way they may play an important role in the regulation of the accumulation of biogenic amines. These data are supported by the work of Nemcsók and Szász (1975) who have shown seasonal changes of Ca^{2+}, K^{+} and Na^{+} ions in the haemolymph of Anodonta.

REFERENCES

Bonting, S.L., Simon, K.A., Howkins, N.M. (1961) Arch Biochem. Biophys. 95, 416-423

Elekes, K. (1976) Acta Biol. Acad. Sci. Hung. 27, 183-189

Gorman, A.L., Marmon, M.F. (1974) J. Physiol. 242, 49-60

Gerschenfeld, H.M. (1973) Physiol. Rev. 53, 1-119

Hiripi, L., Salánki, J. (1971) Annal. Biol. Tihany 38, 31-38

Hiripi, L,, Rakonczay, Z., Nemcsók, J. (1975) Annal. Biol. Tihany 42, 21-28

Hiripi, L., Nezhoff, V., Osborne, N.N. (1976) J. Neurochem. 26, 335-343

Iversen, L.L. (1970) Adv. Biochem. Pharm. 2, 109-132

Iversen, L.L. (1971) Brit. J. Pharm. 41, 571-591

Iversen, L.L. (1973) Brit. Med. Bull. 29, 130-135

Marczynski, T. (1959) Bull. Acad. Pol. Sci. 7, 147-150

Nemcsók, J., Szász, Á.D.J. (1975) Annal. Biol. Tihany 42, 73-80

Nemcsók, J., Rakonczay, Z. (1978) Magyar Kémikusok Lapja 33, 27-31

Osborne, N.N., Hiripi, L., Neuhoff, V. (1975) Biochem. Pharm. 24, 2141-2148

Pollard, H.B., Barker, J.L., Bohr, W.A. Dowdall, M.J. (1975) Brain Res. 85, 23-31

Salánki, J. (1968) In: Symposium on Neurobiology of Invertebrates, pp. 493-501, Akadémiai Kiadó, Budapest, and Plenum Press, New York

Shaskan, E.G., Snyder, S.H. (1970) J. Pharm. Exp. Ther. 175, 404-418

Acta Biochim. Biophys. Hung. 21(1-2), pp. 43-51 (1986)

COMPARISON OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN PARTICLES OF THE DEVELOPING RAT LIVER

T. Tomcsányi, Zs. Bíró, A. Tigyi

Department of Biology, University Medical School, Pécs, Hungary

(Received July 26, 1984)

SUMMARY

hnRNP monoparticles were isolated from livers of newborn (1 day), 7, 10, 11, 14, 21, 28 days old and adult rats, and their sedimentation properties, buoyant densities and protein composition were compared. There were observable differences neither in sedimentation nor in buoyant density among the particles isolated from livers of rats of different ages. The hnRNP particles derived from every stage of ages sedimented in the 30-50S zone of sucrose gradient with a peak at about 38S. Glutaraldehyde fixed particles in CsCl gradients banded at 1.36 g/cm^3 which corresponds to about 80% of protein in the particles. Urea/sodium dodecyl sulfate/polyacrylamide gel electrophoresis of hnRNP proteins demonstrated 19 polypeptides in the 35 000-140 000 M_r range.

Three main polypeptides in hnRNP of every group of animals could be observed with apparent M_r of 39 000, 43 000 and 46 000. In the minor components only quantitative differences could be seen in the different age groups with the exception of a 35 000-M_r polypeptide which appeared on the 11th day after the birth.

INTRODUCTION

The newly synthesized hnRNP exists in the nucleus in the form of ribonucleoprotein particles (for review see, Van Venrooij and Jansen, 1978; Preobrazhensky and Spirin, 1978). According to isolation conditions the heterogeneous nuclear ribonucleoprotein (hnRNP) particles characterized by either a relative low sedimentation coefficient (30-40S), designated as monoparticles or by a higher sedimentation (up to 200S) designated as polyparticles are obtained. In the animals at different stages of the

Akadémiai Kiadó, Budapest

development there are differences in gene activity which initiate distinct cell protein profiles (Church and Brown, 1972; Barth et al., 1982). The factors that control tissue specific gene expression remain unsolved. In this work the physico-chemical properties and the protein composition of the hnRNP monoparticles derived from rat livers of different ages were compared in order to study whether the differences in the gene expression could result in differences in the hnRNP particles. We report here that without changing in the size and protein content of particles the protein composition of the particles undergoes qualitative and quantitative changes during postnatal development of the rat.

MATERIALS AND METHODS

(5-^{3}H)-orotic acid (spec. act 555 GBq/mmol) was obtained from UVVVR (Prague, Czechoslovakia); dithiothreitol from BDH (Poole, England) and all other chemicals from Reanal (Budapest, Hungary).

All buffers contained sucrose were treated with 0.1% diethyl-pyrocarbonate (Fluka, Buchs, Switzerland) to inactivate ribonuclease contamination.

Newborn (1 day), 7, 10, 11, 14, 21, 28 day-old and adult CFY rats received 4 MBq/100 g body weight (^{3}H)-orotic acid one hour before killing. Purified nuclei and nuclear extracts containing hnRNP particles were prepared according to Samarina et al. (1968) with the difference that in the present work all buffers contained 1 mM dithiothreitol. The extracts were centrifuged through 15-30% sucrose gradients. The so called 30S peak fraction and the two adjacent fractions were used for further analysis.

Aliquots of the pooled fractions were fixed with 2.5% glutaraldehyde at 0°C for 4 hours or with 4% formaldehyde for 16 hours. Of the fixed materials 0.3 ml were layered onto CsCl gradients composed 2.2 ml of 1.48 g/cm^3 and 2.2 ml of 1.28 g/cm^3 CsCl solutions and centrifuged at 10°C in an SW 65 Beckman rotor at 40 000 rev/min for 20 hours. The density of every fifth fraction was determined on the basis of its refractive index.

Determination of acid insoluble radioactivity was done as described in a previous paper (Tomcsányi et al., 1981).

hnRNP particles from the pooled fractions were precipitated with two volumes of ethanol (-20°C, 24 hours), washed with 5% and 0.1% trichloroacetic acid, dissolved in 1% SDS/1% 2-mercaptoethanol/ 10 mM sodium phosphate /8 M urea, pH 7.1, heated at 100°C for 5 min and electrophoresed in urea /SDS polyacrylamide gels as described previously (Tomcsányi et al., 1981). The absorbance profiles of the stained gels were recorded at 575 nm using a Joyce-Loebl Chromoscan. Molecular masses of the poly-

peptides were calculated according to Weber and Osborn (1969) using chymotrypsinogen (M_r 25 000) ovalbumin (M_r 43 000) and bovine serum albumin (M_r 67 000) as standards.

The protein concentration of the samples used for electrophoresis was determined by a modified Lowry method (Geiger and Bessman, 1972).

RESULTS

When hnRNP monoparticles derived from livers of rats of different ages were subjected to sucrose gradient centrifugation there were no observable differences in their sedimentation properties. As shown in Fig. 1. hnRNP particles extracted either from newborn (Fig. 1a), one-week- (Fig. 1b), two-weeks-old (Fig. 1c) or adult animals (Fig. 1d) sedimented in the 30-50S zone of the sucrose gradient with a peak at about 38S. This value is in accordance with that of Seifert et al. (1979). Similar results were obtained when hnRNP particles derived from 10, 11, 21 and 28 day-old animals were centrifuged (results not shown).

Particles purified by sucrose gradient centrifugation were fixed with glutaraldehyde in a similar manner as it was done in the case of polysomal (Tomcsányi et al., 1981) and nuclear poly (A)-protein particles (Tomcsányi et al., 1983) and then centrifuged in CsCl gradients to determine their buoyant density. The hnRNP particles derived from animals of all ages, under study, banded in a narrow density range with a peak at 1.36 g/cm^3 (Fig. 2a-e). This value corresponds to a protein content of about 80% (Preobrarhensky and Spirin, 1978) thus the RNA: protein ratio in the rat liver hnRNA particles is 1:4. However, this buoyant density value is lower than the value (1.4 g/cm^3) reported for hnRNA particles derived from different sources fixed with formaldehyde (for review, see Preobrarhensky and Spirin, 1978).

In order to study the reason of this difference a preparation of hnRNP particles from adult rats was divided into two parts. One part was fixed with 2.5% glutaraldehyde, the other with 4% formaldehyde and centrifuged under identical conditions. Fig. 2e shows that the particles fixed with glutaraldehyde banded

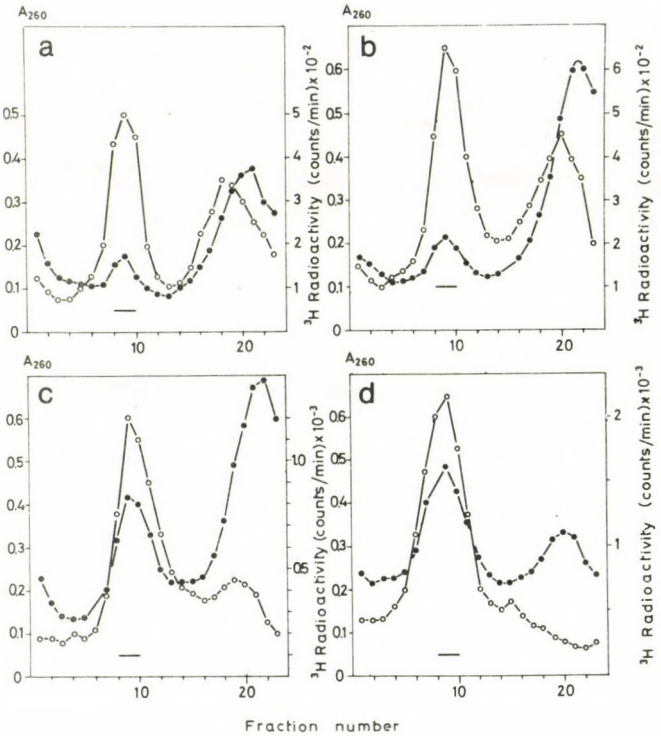


Fig. 1. Sedimentation profiles of nuclear extracts prepared from rat livers of different ages. Nuclear extracts were applied to 15-30% sucrose gradients and centrifuged in a SW 28 rotor at 24 000 rev/min for 17 h. (a) Newborn extract; (b) 1-week-old extract; (c) 2-week-old extract; (d) adult extract. Total fractions were used for determination of absorbance and aliquots of 50 µl for determination of radioactivity. The fractions indicated were pooled and used for further analysis. ●——●, absorbance at 260 nm; o——o, (^{3}H)-acid insoluble radioactivity. The direction of sedimentation is from right to left.

at 1.36 g/cm^3 while the particles fixed with formaldehyde banded at 1.395 g/cm^3 (Fig. 2f) which is identical with the results described previously. It seems, if hnRNP particles are fixed with formaldehyde, as a results of lower fixation effectivity of the formaldehyde (Tomcsányi et al., 1981) the particles lose

about 6-7% of their protein content during the centrifugation in CsCl gradient.

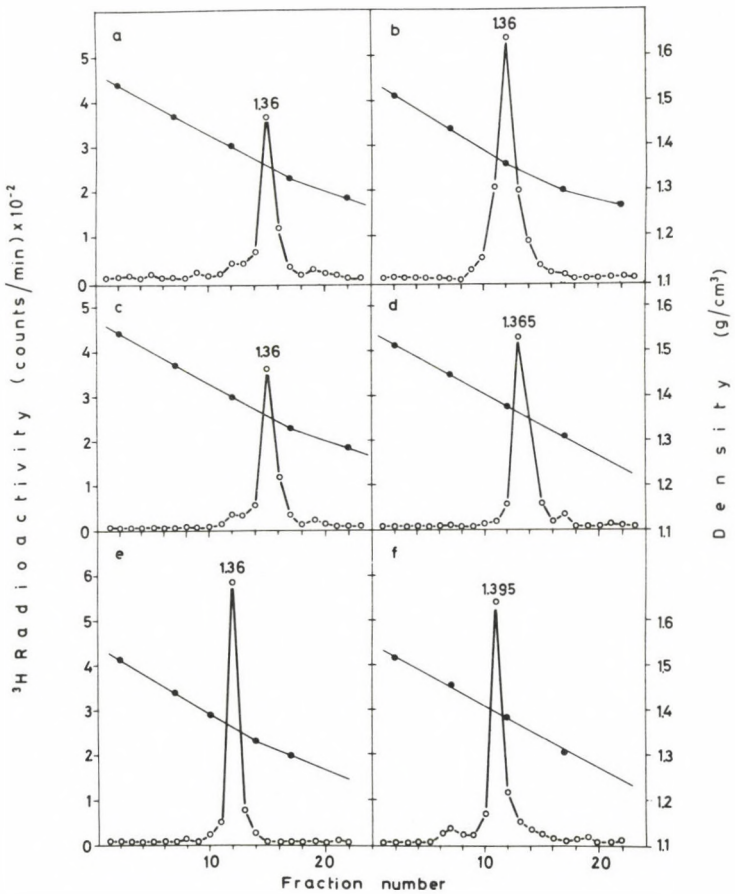


Fig. 2. CsCl gradient centrifugation of hnRNP particles derived from rat livers of different ages. hnRNP particles were fixed with glutaraldehyde or with formaldehyde layered onto CsCl gradients and centrifuged as described in "Methods".
(a) Newborn hnRNP fixed with glutaraldehyde;
(b) 1-week-old hnRNP fixed with glutaraldehyde;
(c) 10-day-old hnRNP fixed with glutaraldehyde;
(d) 2-week-old hnRNP fixed with glutaraldehyde;
(e) adult hnRNP fixed with glutaraldehyde;
(f) adult hnRNP fixed with formaldehyde

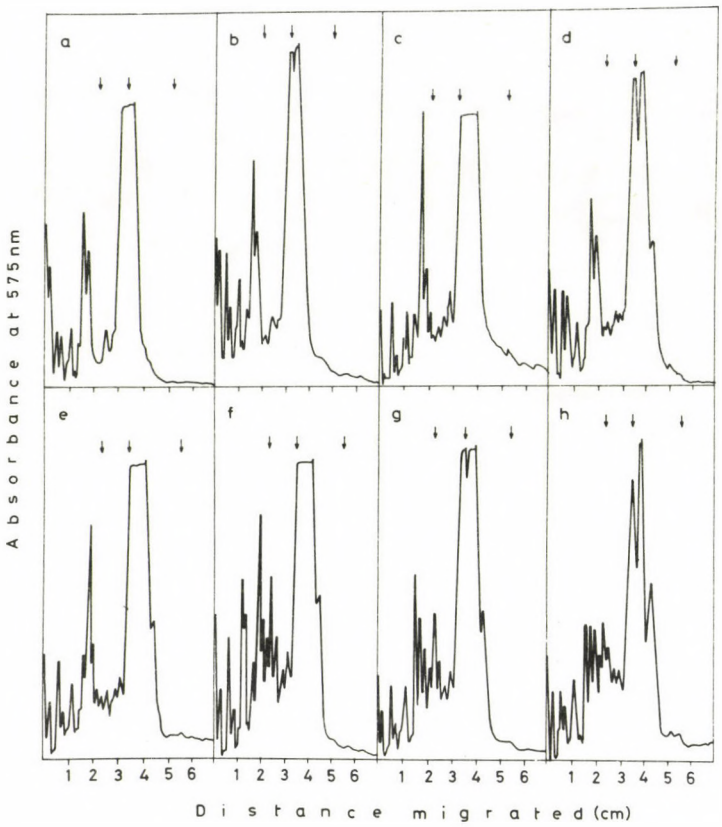


Fig. 3. Stained electropherograms and their densitometric tracings of hnRNP proteins derived from rat livers of different ages. Proteins as described in "Methods" were prepared for electrophoresis and electrophoresed in 8% polyacrylamide gels containing 0.1% SDS and 6 M urea. About 42 µg protein was applied to each gel. (a) Newborn hnRNP; (b) 1-week-old hnRNP; (c) 10-day-old hnRNP; (d) 11-day-old hnRNP; (e) 2-week-old hnRNP; (f) 3-week-old hnRNP; (g) 4-week-old hnRNP; (h) adult hnRNP.
The arrows represent the positions of the markers.

The comparison of the polypeptide composition of hnRNP particles derived from rats of different ages using urea (SDS) polyacrylamide cylindrical gels is shown in Fig. 3. In the particles derived from newborn (Fig. 3a), 7-day- (Fig. 3b)

and 10-day-old animals (Fig. 3c) 18 polypeptides could be observed in the M_r range of 39 000-140 000. Three main polypeptides with apparent M_r of 39 000, 43 000 and 46 000 represent the bulk of hnRNP proteins. The other characteristic feature of the polypeptide patterns of hnRNP from young rats is the relatively large amounts of two minor polypeptides with M_r of 73 000 and 80 000. From the 11th day of age a new, about 35 000-M_r polypeptide appears (Fig. 3d). As the age of the animals (Fig. 3e, f, g) advances their hnRNP polypeptide patterns become more and more similar to the polypeptide pattern of the adult animals (Fig. 3h).

DISCUSSION

The comparison of rat liver hnRNP monoparticles derived from different age groups revealed no differences in their sedimentation and in buoyant density. Therefore, the hnRNP particles in young and adult rats have the same size and protein content. It is interesting to note however that glutaraldehyde, which is a bifunctional reagent, fixed the particles better than formaldehyde, which is a bifunctional reagent, fixed the particles better than formaldehyde did. In CsCl structural proteins dissociate from the particles fixed with formaldehyde. The 1.4 g/cm^3 buoyant density reported for hnRNP particles derived from different sources has been obtained after formaldehyde fixation (for review see, Preobrazhensky and Spirin, 1978), thus this value represents a lower protein content that the real one.

In contrast to the identities of sedimentation coefficients and protein contents of the hnRNP particles of different age groups the electrophoretic separation of the polypeptides by urea/SDS/polyacrylamide gel electrophoresis revealed differences in the protein patterns. According to Molnár and Samarina (1976) rat liver hnRNP polypeptides can be separated into three groups using urea/SDS/polyacrylamide gel electrophoresis. The main group contains polypeptides in the 35 000-50 000 M_r range. The minor polypeptides can be divided into two groups, the one contains polypeptides up to 100 000-M_r, the other contains

polypeptides higher than M_r of 100 000.

Prior to 11th day of age the 35 000-M_r polypeptide lacks the first protein group. The other polypeptides are present and the minor polypeptides differ in their quantities. In mouse liver no changes were found in the protein composition of the hnRNP particles during tissue development (Talley-Brown, 1977) but the particles studied derived only from 1-, 3-, 8-week-old animals. In amphibian liver quantitative changes were shown in the protein composition of nuclear RNP (Maxwell and Fisher, 1979). It has also been shown that after 10 weeks of feeding of the carcinogenic dye 3'-methyl-4-dimethyl-aminoazobenzene the 42 000-M_r polypeptide completely disappeared from rat liver hnRNP particles (Patel and Holoubek, 1977a).

Although several models have been proposed for the structure of hnRNP particles (Samarina et al., 1968; Sekeris and Niessing, 1975; Heinrich and Northemann, 1981; LeStourgeon et al., 1981) the exact structure of the particles is not yet known. The particles have a number of enzyme activities, most of them are possible processing enzymes (for review see, Jeanteur, 1981) but which enzyme acitivities belong to the individual hnRNP proteins have not yet been identified.

It has been suggested that the proteins with M_r of 30 000-45 000 from a core in the particles (Patel and Holoubek, 1977b; Heinrich and Northemann, 1981) to which hnRNA and various proteins can be associated. These associated proteins could differ from particle to particle according to the type of RNA bound to the core particle, or even the same core particle could be associated with dissimilar proteins at various stages of RNA processing (Patel and Holoubek, 1977b). Since the liver during its development undergoes specific changes in the gene activity (Church and Brown, 1972; Barth et al., 1982), it seems the changes are represented not only at mRNA level in the cytoplasm but also at RNP level in the nucleus. Our results also suggest that hnRNP particles are dynamic structures which can alter their protein composition at different stages of the development without any change in their size and protein content.

REFERENCES

Barth, R.K., Gross, K.W., Gremke, L.C., Hastie, N.D. (1982) Proc. Natl. Acad. Sci. USA 79, 500-504

Church, R.B., Brown, J.R. (1972) in Biochemistry of Cell Differentiation (Monroy, A., Tsanev, R. eds.) FEBS Symposium vol. 24, pp. 99-107, Academic Press London, New York

Geiger, P.J., Bessmann, S.P. (1972) Anal. Biochem. 49, 467-473

Heinrich, P.C., Northemann, W. (1981) Mol. Biol. Rep. 7, 15-24

Jeanteur, P. (1981) in The Cell Nucleus (Busch, H. ed.) vol. IX., pp. 145-170, Academic Press, New York

LeStourgeon, W.M., Lothstein, L., Walker, B.W., Bayer, A.L. (1981) in The Cell Nucleus (Busch, H. ed.) vol. IX., pp. 49-87, Academic Press, New York

Maxwell, E.S., Fischer, M.S. (1979) Biochim. Biophys. Acta 562, 319-330

Molnár, J., Samarina, O.P. (1976) Mol. Biol. Rep. 3, 195-202

Patel, N.T., Holoubek, V. (1977a) Chem. Biol. Interactions 19, 303-316

Patel, N.T., Holoubek, V. (1977b) Biochim. Biophys. Acta 474, 524-535

Preobrazhensky, A.A., Spirin, A.S. (1978) Prog. Nucleic Acid Res. Mol. Biol. 21, 1-38

Samarina, O.P., Lukanidin, E.M., Molnár, J., Georgiev, G.P. (1968) J. Mol. Biol. 33, 251-263

Seifert, H., Scheurlen, M., Northemann, W., Heinrich, P.C. (1979) Biochim. Biophys. Acta 564, 55-66

Sekeris, C.E., Niessing, J. (1975) Biochem. Biophys. Res. Commun. 62, 642-650

Talley-Brown, S. (1977) Fed. Proc. 36, A3474

Tomcsányi, T., Komáromy, L., Tigyi, A. (1981) Eur. J. Biochem. 114, 421-428

Tomcsányi, T., Molnár, J., Tigyi, A. (1983) Eur. J. Biochem. 131, 283-288

Van Venrooij, W.J., Janssen, D.B. (1978) Molec. Biol. Rep. 4, 3-8

Weber, K., Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

Acta Biochim. Biophys. Hung. 21(1-2), pp. 53-58 (1986)

EFFECT OF 3-METHYLCHOLANTHRENE ON THE PROTEIN COMPOSITION OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN PARTICLES IN DEVELOPING RAT LIVER

T. Tomcsányi, Zs. Bíró, J. Szeberényi, A. Tigyi

Department of Biology, University Medical School, Pécs, Hungary

(Received July 26, 1984)

SUMMARY

Nine-day-old rats were treated with a single 100 mg/kg body weight intraperitoneal dose of 3-methylcholanthrene (3-MC). 24, 48, 72 and 120 h later the protein composition of the heterogeneous nuclear ribonucleoprotein particles derived from the liver was analysed. The analysis was performed by urea-sodium dodecyl sulphate polyacrylamide gel electrophoresis. The protein composition of the hnRNP particles was compared to that derived from control animals of similar ages. The 35 000-M_r polypeptide which is normally absent found in the hnRNP particles of the young rats prior to the 11th day of life appears on the 10th day after 24 h 3-MC exposition. In addition, the minor polypeptide components of the particles were also found in higher quantity. The polypeptide pattern of the hnRNP particles derived from treated animals resembled those of the older animals but not those of controls of similar ages. Thus, the apparent accelerating effect of 3-MC on the liver development is also valid at the level of hnRNP particles.

INTRODUCTION

In rats, a single injection of the polycyclic hydrocarbon, 3-MC, rapidly stimulates the synthesis of cytochrome P-450 (Nebert, 1978) which is a member of NADPH or NADH dependent membrane-bound multicomponent enzyme systems (Sato and Imura, 1978). The induction of the enzymes is regulated by the Ah locus (Nebert, 1978). It has been shown that 3-MC produces elevated activities of RNA polymerases (Kleberg et al., 1982) and initiation factors of protein synthesis (Hopkinson et al., 1974) as well as an increase in RNA synthesis (Bresnick et al., 1968). 3-MC has also been reported to promote the development of the

liver (Klinger et al., 1978).

In a previous paper (Tomcsányi et al., 1985) we have described that the liver hnRNP particles, without changing their size and protein content, undergo qualitative and quantitative changes in their protein composition during postnatal development. In the present study we have isolated hnRNP particles from the liver of young rat pretreated with 3-MC and compared their protein composition to the particles of untreated animals of similar ages. It is shown that the polypeptide patterns of the hnRNP particles derived from 3-MC treated rats resemble those of the older animals and not those of the controls of similar ages.

MATERIALS AND METHODS

Chemicals

Dithiothreitol was obtained from BDH (Poole, England) while all other chemicals were from Reanal (Budapest, Hungary).

All buffers containing sucrose were treated with 0.1% dimethylpyrocarbonate (Fluka, Buchs, Switzerland) to inactivate ribonuclease contamination.

Animals

Nine-day-old CFY rats received a single intraperitoneal dose of 3-MC (100 mg/kg body weight). After 24, 48, 72 and 120 h the animals were sacrificed and the hnRNP particles were prepared from their livers.

Preparation of hnRNP particles

Purified nuclei and nuclear extracts containing hnRNP particles from the livers were prepared according to Samarina et al. (1968), except that in the present work all buffers contained 1 mM dithiothreitol. The nuclear extracts were centrifuged through 15-30% sucrose gradients. The 30S peak and the two adjacent fractions were used for further analysis.

Polyacrylamide gel electrophoresis of hnRNP proteins

hnRNP particles from the pooled fractions were precipitated with two volumes of ethanol (-20°C, 24 h), washed with 5% and 0.1% trichloroacetic acid, containing 1% SDS, 1% 2-mercaptoethanol, 10 mM sodium phosphate and 8 M urea, pH 7.1, and heated at 100°C for 5 min then electrophoresed in urea/SDS polyacrylamide gels as described previously (Tomcsányi et al., 1981). The absorbance profiles of the stained gels were recorded at 575 nm using a Joyce-Loebl Chromoscan. Molecular masses of the polypeptides were calculated according to Weber and Osborn using chimotrypsinogen (M_r 25 000), ovalbumin

(M_r 43 000) and bovine serum albumin (M_r 67 000) as standards.

Protein assay

The protein concentration of the samples used for electrophoresis was determined by a modified Lowry method (Geiger and Bessman, 1972).

RESULTS AND DISCUSSION

It has previously been found that the 35 000-M_r "core" polypeptide is normally absent from rat liver hnRNP particles prior to the 11th days of ages (Tomcsányi et al., 1985). As shown in Fig. 1, 24 h after the administration of 3-MC to nine-day-old rats, the 35 000-M_r polypeptide appears in the hnRNP particles of the liver (Fig. 1b). In addition, the increase in the relative amounts of the 94 000-M_r, the 100 000-M_r, and the 105 000-M_r can be observed in the hnRNP particles of the 24 h 3-MC-treated animals (Fig. 1b). Forty-eight hours after 3-MC injection the amount of the 94 000-M_r polypeptide is increased (Fig. 1d) in comparison to the polypeptide pattern of the control animals (Fig 1c). The polypeptide pattern of hnRNP particles derived from 10-day-old, 24 h 3-MC treated rats (Fig. 1b) resembles that of 14-day-old untreated animals (Fig. 1g). By 72 and 120 h, the polypeptide patterns are very similar to each other (Fig. 1f and h) and resemble that of 21-day-old untreated animals (result not shown). Thus, 72 h after 3-MC treatment the hnRNP pplypeptide pattern does not undergo further changes but remain the same in the period studied (120 h).

It should be noted that both the sedimentation properties and the protein content of the hnRNP particles derived from 3-MC-treated animals are similar to those of the untreated animals (results not shown).

The most remarkable effects of 3-MC treatment on the hnRNP particles of young rats are the appearance of the 35 000-M_r "core" polypeptide one day earlier than normal and an increase in the amount of the minor polypeptides. At present, the function of the minor polypeptides is unknown but they are authentic components of hnRNP as proved by RNA-

protein cross-linking in vivo (Economidis and Pederson, 1983).

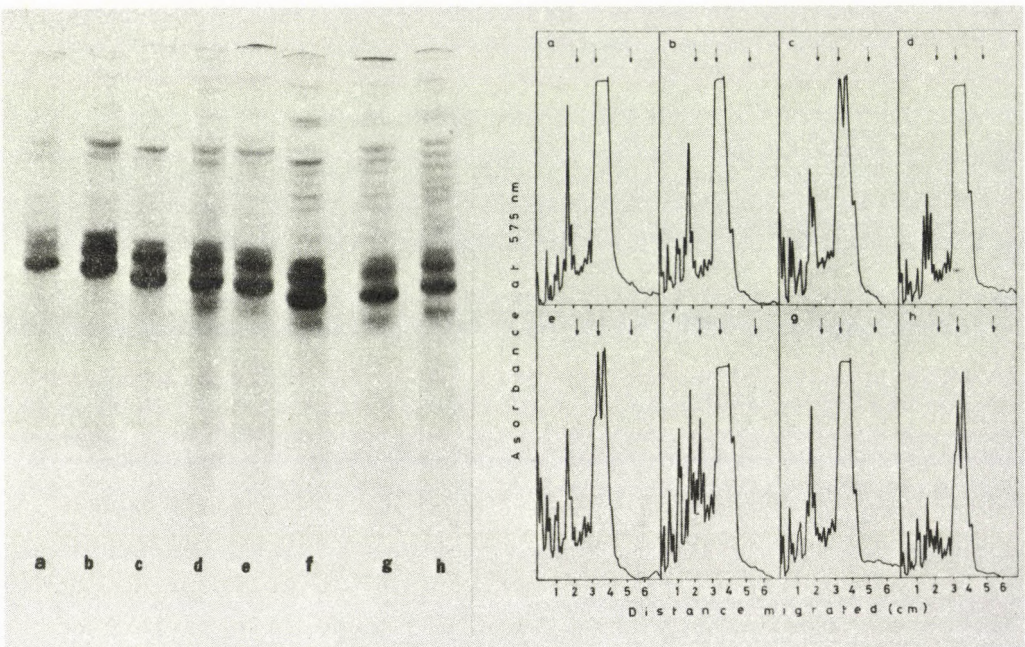


Fig. 1. Stained gels and their densitometric tracings of the portein moiety of heterogeneous ribonucleoprotein particles derived from the livers of 3-methylcholanthrene treated and untreated rats separated by urea/sodium dodecyl sulphate polyacrylamide gel electrophoresis. The particles were extracted with 0.1 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$ and purified by centrifugation through 15 to 30% sucrose gradient.
(a) 10-day-old rats, untreated, 42 µg protein,
(b) 10-day-old rats, 24 h 3-MC-treated, 42 µg protein,
(c) 11-day-old rats, untreated, 42 µg protein,
(d) 11-day-old rats, 48 h 3-MC-treated, 41 µg protein,
(e) 12-day-old rats, untreated, 40 µg protein,
(f) 12-day-old rats, 72 h 3-MC-treated, 43 µg protein,
(g) 14-day-old rats, untreated, 42 µg protein,
(h) 14-day-old rats, 120 h 3-MC treated, 40 µg protein

The effect of the xenobiotic on the hnRNP particles is reflected, therefore, by the changes in the quantities of the minor polypeptides. Moreover, the effect of 3-MC on the hnRNP particles is longer than the effects on RNA polymerase activity and synthesis of cytochrome P-450 mRNA. In young rats the elevated RNA polymerase activities reach a maximum 24 h after 3-MC treatment, and 96 h after the level of RNA polymerase activities is the same as in the controls (Kleeberg et al., 1982). In adult rats the number of cytochrome P-450 mRNA molecules is the highest 15 h after the treatment, after then there is a considerable decrease (Bresnick et al., 1981). In contrast, the hnRNP polypeptide pattern remains altered 120 h after the treatment in comparison to the control.

Since the polypeptide patterns of the hnRNP particles derived from 3-MC treated animals resemble those of the older animals and not those of the controls of similar ages, the apparent acceleration of 3-MC on liver development is also valid at the level of hnRNP particles.

Acknowledgement

The skilful technical assistance of Mrs Mária Schaffer is acknowledged. Authors are indebted to Mr. Wayne Cotton for his help in the preparation of the manuscript.

This work was supported by a grant from the Hungarian Medical Science Council (05/3-04/248).

REFERENCES

Bresnick, E., Synerkohn, M.E., Tizard, G.T. (1968) Molec. Pharm. 4, 218-223

Bresnick, E., Brosseau, M., Lewin, W., Reitz, L., Ryan, D., Thomas, P.E. (1981) Proc. Natl. Acad. Sci. USA, 48, 4083-4087

Economidis, J.V., Pederson, T. (1983) Proc. Natl. Acad. Sci. USA, 80, 1599-1602

Geiger, P.J., Bessman, S.P. (1972) Anal. Biochem. 49, 467-473

Hopkinson, J., Prichard, P.M., Bresnick, E. (1974) Biochim. Biophys. Acta 374, 375-383

Kleeberg, U., Szeberényi, J., Juhász, P., Tigyi, A., Klinger, W. (1982) Biochem. Pharm. 31, 1063-1067

Klinger, W., Mueller, D., Kleeberg, U. (1978) in The Induction of Drug Metabolism (Estabrook, R.W., Lindenlaub, E. eds.) pp. 517-544, F.K. Schattauer Verlag, Stuttgart, New York

Nebert, D.W. (1978) in The Induction of Drug Metabolism (Estabrook, E.W., Lindenlaub, E. eds.) pp. 419-452, F.K. Schattauer Verlag, Stuttgart, New York

Samarina, O.P., Lukanidin, E.M., Molnár, J., Georgiev, G.P. (1968) J. Mol. Biol. 33, 251-263

Sato, R., Imura, T. (eds.) Cytochrome P-450, Academic Press, New York (1978)

Tomcsányi, T., Komáromy, L., Tigyi, A. (1981) Eur. J. Biochem. 114, 421-427

Tomcsányi, T., Bíró, Zs., Tigyi, A. (1985) Acta Biochim. Biophys. Hung. (in press)

Weber, K., Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

Acta Biochim. Biophys. Hung. 21(1-2), pp. 59-65 (1986)

THE EFFECT OF α-DIFLUOROMETHYLORNITHINE ON ORNITHINE DECARBOXYLASE ACTIVITY IN COMPENSATORY GROWTH OF MOUSE LUNG

Susan Bardócz, Susan Tatár-Kiss[§] and P. Kertai[§]

Department of Biochemistry and Department of Hygiene and Epidemiology[§], University Medical School of Debrecen, H-4012 Debrecen, POB 6, Hungary

(Received February 10, 1986)

SUMMARY

After removing the left upper lobe, the remaining lung of mice undergoes compensatory growth. Increased ornithine decarboxylase activity is characteristic of the process. The effect of α-difluoromethylornithine was studied on the ornithine decarboxylase (E.C.4.1.1.17.) activity and on the lung growth in mice after lobectomy. Although a single injection of α-difluoromethylornithine inhibited ornithine decarboxylase activity, the compensatory growth of lung still occurred in spite of continuous α-difluoromethylornithine treatment. This suggests that polyamines, indispensable for compensatory growth, may be supplied by other sources.

INTRODUCTION

Following the injuries of mammalian organs, regeneration appears to make up for the function of the deficient part by the organism itself. Hepatectomy is followed by regeneration, whereas the injuries of kidney and heart are followed by hypertrophy. High ornithine decarboxylase (E.C.4.1.1.17) activity was observed during both processes.

It is still not obvious, how the remaining lung is able to grow after pulmonectomy or lobectomy. Compensatory lung growth was observed in female BALB/c mice after the surgical removal of the left upper lobe, so called lobectomy (Tatár-Kiss

Abbreviations: ODC, ornithine decarboxylase (E.C.4.1.1.17.); DFMO, α-difluoromethylornithine

et al., 1984). The mass of the remaining lobes is increasing rapidly, reaching the total mass of the original organ within two weeks after operation. An enhanced ODC activity is accompanied by the process. The participation of the individual lobes is disproportionate, each exhibits different growth rate and ODC activity (Tatár-Kiss et al., 1984).

The question arises whether the increased ODC activity plays an essential role in initiating growth, or it influences the process only indirectly. The effect of α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ODC (Metcalf et al., 1978), was studied on lung regeneration by measuring the growth rate and ODC activity in the remaining lobes after lobectomy.

MATERIALS AND METHODS

Animal experiments

The experiments were carried out with inbread BALB/c female mice of 18-20 g weight, kept on conventional laboratory environment, fed on semisynthetic diet (LATI, Gödöllő, Hungary). The animals had free access to tap water or water containing DFMO. The investigated animals were devided into 4 groups. The experimental group was lobectomized and treated with DFMO. Three different control groups were used: a.) sham-operated; b.) sham-operated and treated with DFMO; c.) lobectomized without DFMO treatment.

To study the effect of DFMO on ODC activity mice were injected once with 400 mg/kg body weight DFMO i.p., and two hours later lobectomized or sham-operated as described earlier (Tatár-Kiss et al., 1984). The controls were injected with the same volume of saline at the same time, then lobectomized or sham-operated.

Compensatory lung growth was studied in the following way. The mice were lobectomized or sham-operated, then kept on water containing 2% DFMO (w/v) for two weeks. The controls were given tap water.

Chemicals

DFMO was kindly provided by Merrell Dow Research Institute Strasbourg Center (France). Pyridoxal 5-phqsphate and dithiothreitol were purchased from Serva (Heidelberg, FRG). D, L-1 ^{14}C-ornithine.HCl (specific activity: 1.57 mCi/mmol) was obtained from the Isotope Institute of Hungarian Academy of Sciences (Budapest, Hungary). All other chemicals were Reanal (Budapest, Hungary) products of analytical grade.

Tissue preparation and assay of ODC activity

After cervical dislocation the lungs were perfused in situ with physiological saline, then removed and weighed. The samples were homogenized in 4 volumes of ice-cold 100 mM phosphate buffer, pH 7.2, containing 5 mM dithiothreitol and 5 mM pyridoxal 5-phosphate, then centrifuged (50 000 x g, 30 min, 5°C). The enzyme activity was determined in the supernatant by the modified method of Russell and Snyder (1968), as described earlier (Bardócz et al., 1984). The protein content of the samples was measured according to Markwell et al. (1978).

RESULTS

Effect of DFMO on ODC activity

The highest ODC activity in the remaining lung of female BALB/c mice was measured 4 hours after removing the left upper lung lobe (Tatár-Kiss et al., 1984), therefore this time interval was also chosen for measuring the effect of DFMO on ODC activity in compensatory lung growth.

The mice were treated with a single i.p. injection of 400 mg/kg DFMO two hours prior lobectomy or sham-operation. The ODC activity measured in the individual lung lobes 4 hours after surgery was compared to that of untreated controls (Fig. 1). In the various lobes of sham-operated or DFMO treated groups the enzyme activity did not differ much, however there were differences in lobectomized groups.

Lobectomy was followed by a 2 to 5-fold increase in ODC activity of the individual lobes, while it was decreased significantly to 5-10% of the original value after DFMO treatment. The results show that a single dose of DFMO readily inhibits ODC activity, though the remaining activity is still 20-60% of the values measured originally.

Effect of DFMO on compensatory growth

The lobectomized lung reaches the total weight of the original one within two weeks (Tatár-Kiss et al., 1984). We allowed the sham-operated and lobectomized mice to drink water containing 2% DFMO during this period. This dose is similar to that used for inhibiting ODC activity since an animal drinks 5-6 ml water daily.

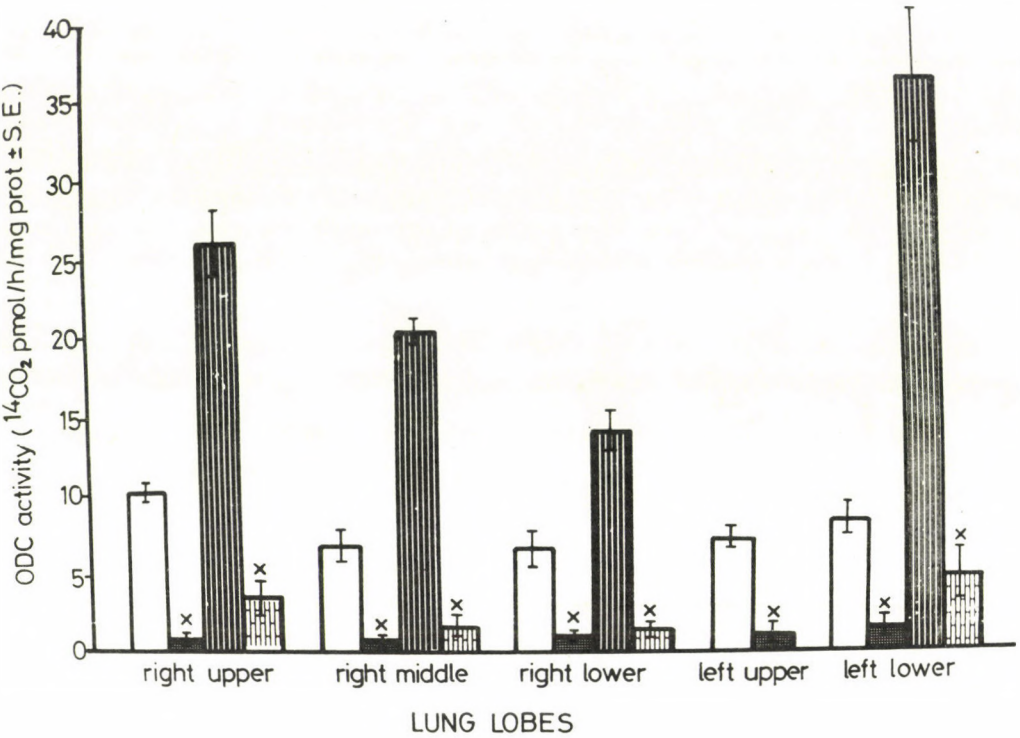


Fig. 1. Effect of DFMO treatment and lobectomy on the ODC activity of lung lobes four hours after operation. Female BALB/c mice were treated with DFMO (400 mg/kg; i.p.), and lobectomized two hours later (column ▨). Controls: (□) sham-operated; (■) sham-operated and DFMO treated; (▨) only lobectomized mice. The values are Mean ± S.E. for 5 animals per groups. Asterisks indicate significant differences between DFMO treated and control mice, $p < 0.05$.

The mass of lung lobes measured two weeks after continuous DFMO treatment in the experimental group and controls is shown in Fig. 2. The remaining lung reaches the total weight of the original organ within two weeks in both lobectomized groups. This indicates that the compensatory growth goes on in spite of DFMO treatment. The data also suggest that DFMO may interfere with the normal growth regulating process, since slightly higher values were measured after sham-operation in

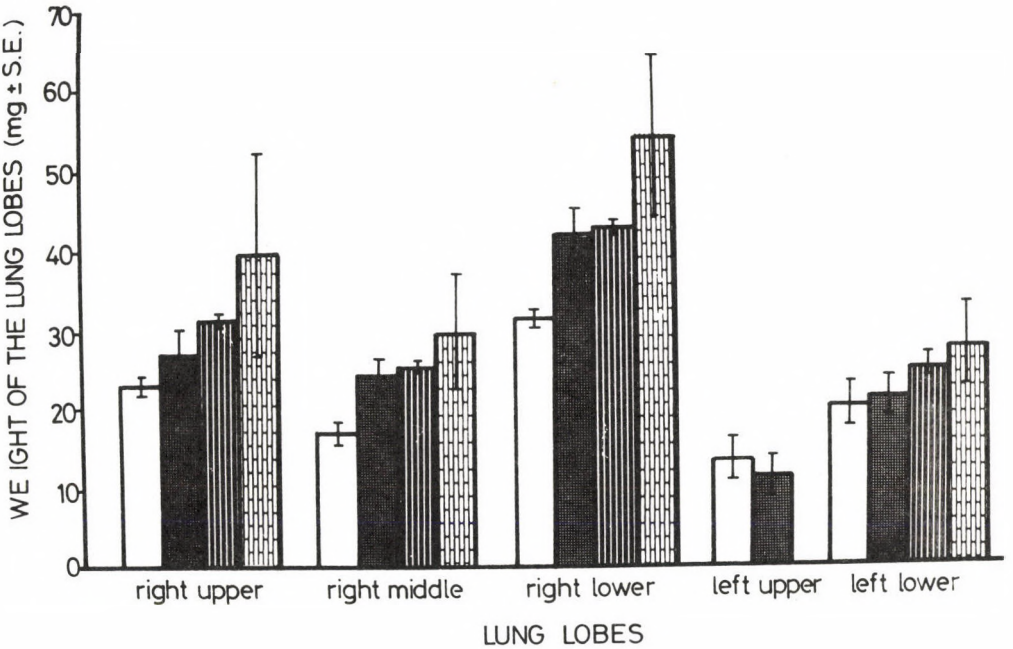


Fig. 2. Effect of DFMO treatment and lobectomy on the mass of individual lung lobes.Female BALB/c mice were treated with 2% DFMO containing water for two weeks after lobectomy (column ▨). Controls: (□) sham-operated; (■) sham-operated and DFMO treated; (▨) only lobectomized mice. The values are the Mean ± S.E. for 5-15 animals per group.

the DFMO treated groups that in the respective controls, but the differences are not significant.

The results appear to indicate clearly that the increase in ODC activity is rather a useful marker than an essential requirement of the compensatory lung growth.

DISCUSSION

During the past two years it has been confirmed that DFMO inhibits ODC activity in mammals, however it does not prevent

tomour growth in animals and is ineffective as an anticancer drug in humans (Marton et al., 1981; Danzin et al., 1982); Fozard et al., 1982). It is also clear from the present data that DFMO cannot prevent "regulated" growth, such as the compensatory growth of lung.

The reason for increased ODC activity is most probably to promote polyamine production. The results does not necesarily imply that an increased ODC activity is required for compensatory growth, though it is characteristic of the process. According to our present knowledge, polyamines are obligatory for cell proliferation and growth. We have to assume that in mammals there may be other pathways, different from the biosynthetic route to provide polyamines. The lungs, besides its active metabolism, have a very intensive transport system (Gaudreault et al., 1984). The animals can take up polyamines from food, which may or may not reach the internal organs. Moreover, symbiotic microorganisms of mammals can synthetize polyamines that might be taken up by the intestinal transport system. This idea is supported by the findings that the polyamine uptake is increased by cells after DFMO treatment (Seidenfeld and Marton, 1979) and the cadaverine content, a polyamine of microorganisms, is enhanced in the circulation.

The interconversion of polyamines (Seiler and Bolkeneius, 1985; Tabor and Tabor, 1984) can also supply these compounds on a metabolic route, though this pathway is also utilizing polyamines simultaneously. The interconversion may reorganize the polyamine pools by changing their distribution in the body.

Acknowledgements

This work was supported by the grants of the Hungarian Ministry of Health (2-10-0401-01-2/k) and the Hungarian Academy of Sciences.

Thanks are due to the Merrell Dow Research Institute, Strasbourg Center, for providing DFMO for the experiments. The technical assistance of Miss Zita Lukács, Miss Judith Nagy and Miss Judith Virányi is greatly acknowledged.

REFERENCES

Bardócz, S., Rády, P., Várvölgyi, Cs. and Karsai, T. (1984) Anticancer Res. 4, 59-62

Danzin, C., Claverie, N.I., Wagner, J., Grove, J. and Koch-Weser, J. (1982) Biochem. J. 202, 175-181

Fozard, J.R. and Prakash, N.J. (1982) Naunyn-Schmiedeberg's Arc. Pharmacol. 320, 72-77

Gaudreault, P., Karl, P.I. and Friedman, P.A. (1984) Drug Metabolism and Disposition 12, 550-552

Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206-210

Marton, L.J., Levin, V.A., Hervatin, S.J., Koch-Weser, J., McCann, P.P. and Sjoerdsma, A. (1981) Cancer Res. 41, 4426-4431

Metcalf, B.W., Bey, P., Danzin, C., Jung, M.J., Casara, T. and Vevert, J.P. (1978) J. Am. Chem. Soc. 100, 2551-2553

Russell, D.H. and Snyder, S.H. (1968) Proc. Natl. Acad. Sci. USA 60, 1420-1427

Seidenfeld, J. and Marton, L.J. (1979) Biochem. Biophys. Res. Commun. 86, 1192-1198

Seiler, N. and Bolkeneius, F.N. (1985) Neurochem. Res. 10, 529-544

Tabor, C.W. and Tabor, H. (1984) Ann. Rev. Biochem. 53, 749-790

Tatár-Kiss, S., Bardócz, S. and Kertai, P. (1984) FEBS Letters 175, 131-134

Acta Biochim. Biophys. Hung. 21(1-2), pp. 67-80 (1986)

STEROID SPECTRUM IN HUMAN URINE AS REVEALED BY GAS CHROMATOGRAPHY. VI. PREGNANEDIOL EXCRETION VALUES AND 3β-HYDROXY-Δ5-STEROID DEHYDROGENASE ACTIVITY OF GIRLS AT DIFFERENT STAGE OF DEVELOPMENT

L. Kecskés, Zsuzsanna Juricskay, Márta Szécsényi, Edit Palotás

Central Laboratory, Medical University, Pécs, Hungary

(Received March 24, 1983)

SUMMARY

Pregnenediol excretion in the urine of girls aged 2-14 years was found to be 0.13 ± 0.17 mg/day. Before puberty the amount of pregnanediol excreted was low but a marked increase could be observed during puberty. In the case of premenarchal girls this increase correlated well with increasing body weight, while in the presence of cycle ovarian function (anovulational cycles) it exceeded the expected level.

The activity of 3β-hydroxy-Δ5-steroid dehydrogenase was assayed in vivo indirectly, by measuring pregnanediol (pregnenediol and androsterone + ethiocholanone) DHA ratios from urine. A composition of the two ratios indicated that the utilization of the endogenous C_{21} intermediate was one order of magnitude less than that of the C_{19} intermediate. To interprete this finding it is assumed that the Δ5-3β-HSD of the liver is on allotype of the adrenal enzyme with a reduced activity, towards the C_{21} intermediate. The biological significance of this presumption is also discussed.

Abbreviations: DHA, dehydroepiandrosterone; P 1-4, stages of puberty; 3α-HSD, 3α-hydroxy-steroid dehydrogenase; 3α-hydroxy-steroid: $NAD(P)^+$ oxidoreductase (E.C. 1.1.1.50.); Δ5-3β-HSD, 3β-hydroxy-Δ5-steroid dehydrogenase; 3β-hydroxy-Δ5-steroid: NAD^+ 3-oxidoreductase (E.C. 1.1.1.145.): Δ4-α-reductase, 4,5α-dihydrocortisone: $NADP^+$ Δ4-oxidoreductase (E.C. 1.3.1.4.): Δ4-β-reductase, 4,5β-dihydrocortisone: $NADP^+$ Δ4-oxidoreductase (E.C. 1.3.1.3.); Δ4,5-isomerase, 3-oxosteroid-Δ4-Δ5-isomerase (E.C. 5.3.3.1.)

Akadémiai Kiadó, Budapest

INTRODUCTION

Puberty is a highly dynamic period in human ontogenesis. The androgene production of adrenals rapidly increases (adrenarche) and this is thought to be responsible for the development of pubic hair in girls (Albright et al., 1942). Parallel with adrenarche a continous layer of reticular cells in the adrenal cortex is formed (Dohm, 1973). Numerous data indicate that in young children the retarded ontogenesis of the zona reticularis is responsible for the low androgene values and for the low C-17,21 lyase activity. The plasma level of pregnenolone (Apter and Vihko, 1977), as well as pregnenediol excretion in the urine (Kecskés et al., 1980) also increase parallel with the rise in the androgene level.

In puberty the elevation of androgene and pregnenolone levels in the plasma, as well as that of the excretion of metabolites per kilogramm body weight indicate the formation of the new structure, the zona reticularis. In contrast, the plasma cortisol level is stable and the increase of excreted cortisol metabolites in the urine does not exceed the rate of increase in body weight.

The progesterone level (Apter and Vihko, 1977) and pregnanediol excretion, per kilogramm, body weight show only a marginal rise during puberty (Kecskés, 1982) but increase significantly after the onset of the menstruational cycle indicating the ovarian origin of progesterone.

In the present work, besides pregnanediol excretion, we indirectly determined the activity of 3β-hydroxy-Δ5-steroid dehydrogenase from the ratio pregnanediol/pregnanediol as determined from the urine. In vivo measurements of this kind have not been reported so far in the literature. On the basis of androsteron + ethiocholane (dehydroepiandrosterone, as well as pregnanediol) pregnenediol ratios we compared the 3β-hydroxy-Δ5-(C_{19} and C_{21})-steroid dehydrogenase activities and found that the utilisation of endogenous $C_{21}O_2$ intermediates in the body is one order of magnitude less than that of the $C_{19}O_2$ steroids. We shall also discuss the biological interpretation of this great difference.

MATERIALS AND METHODS

24 hour urine was collected from 29 healthy girls on 34 occasions. The age distribution was the following: between 2-7 years: 12 girls (young children's group); 7-10 years: 9 girls (pre-puberty group); 10-14 years: 13 girls (puberty group). The latest group was subdivided according to puberty stages: 4 girls were in P-2, 5 girls in P-3 and 4 girls in P-4. Urine from the P-4 group was collected between the 15th and 30th day after their latest period. We have already reported on the cumulative values of adranal androgenes and their metabolites (DHA, androsteron, ethiocholanon), representing as the $C_{19}O_2$-steroid group, as well as on pregnenediol excretion in various stages of somatic development (Kecskés et al., 1982). Now, in addition, we give a detailed account of the DHA values from these measurements.

Extraction of steroids, formation of derivatives and gas-chromatography were performed in a two-column-two-channel system (Kecskés et al., 1981), which is a modified version of our previous method (Kecskés and Juricskay, 1975) made suitable to measure 16-hydroxy steroids.

RESULTS

The pregnanediol excretion (output) in girls aged between 2 and 14 years was 0.13 ± 0.17 mg/day (mean of 34 cases), with a minimum of 0.0, and a maximum of 0.65 mg. Table 1 demonstrates the results according to age distribution. In 10-14 year old girls during puberty a significant elevation of pregnanediol excretion was observed. In the following these results were analysed in two groups. The pregnenediol excretion of girls in the second and third stage of puberty was compared with that of the already menstruating fourth group. The rise in pregnenediol excretion of girls reaching the 2nd-3rd stage of puberty was significant (P 0.02, not indicated in Table 1), as compared to the values of the 7-9 year group. Moreover, this tendency continued in P-4 group (Table 2).

Puberty is characterised by a rapid increase in body weight and a paralelly occurring general development of endocrine organs in order to maintain homeostasis. Therefore it seemed plausible to relate the pregnanediol values to body weight too, because in this way the general growth factors could be separated from those connected with specific gonado maturation. Pregnanediol, androsterone + ethiocholane and DHA

Table 1. Excretion of pregnanediol by healthy girls of different ages (mg/24 h)

Age (year)	n	mean ± SD	P	Range
2 - 6	12	0.05 ± 0.03	n.s.	0.00 - 0.12
7 - 9	9	0.06 ± 0.03	< 0.02	0.02 - 0.12
10 - 14	13	0.26 ± 0.21		0.07 - 0.65

Table 2. Excretion of pregnanediol by healthy girls at different stages of puberty (mg/24 h)

Puberty stage	n	mean ± SD	P	Range
II - III	9	0.17 ± 0.12	< 0.02	0.07 - 0.43
IV	4	0.47 ± 0.23		0.14 - 0.65

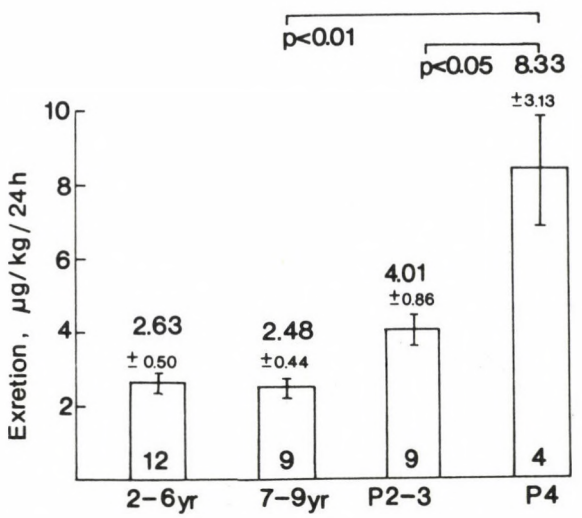


Fig. 1. Excretion values in µg/kg/day of pregnanediol according to age group distribution and puberty stages.

Table 3. Excretion of pregnanediol, androsterone + etiocholanolone and DHA by healthy girls of different ages (µg/kg/24 h)

Age (year)	n	Pregnanediol mean±SD	Range	P	Androsterone + Etiocholanolone mean±SD	Range	P	DHA mean±SD	Range	P
2 - 6	12	2.63±1.72	0.0-6.2	n.s.	3.1±1.8	1.2-7.3	<0.01	0.88±0.43	0.3-2.0	n.s.
7 - 9	9	2.48±1.16	1.8-4.7	n.s.	7.6±4.9	2.6-16.8	<0.01	1.10±0.47	0.5-2.0	<0.02
10 - 14	13	6.02±3.48	1.5-11.0		30.2±17.2	7.0-57.2		3.08±2.14	0.6-8.4	

values, related to body weight and arranged by age groups, are depicted in Table 3. Although the pregnanediol (body weight ratio in the 10-14 year group is doubled as compared to the preceeding (7-9 year) one, this increase cannot be regarded as significant, due to the large increase in the S.D. The 10-14-year group, therefore, was divided into two parts according to the stage of puberty and the mean±S.D. values for both the age- and puberty groups are shown in Fig. 1. The elevation of pregnenediol output in the P-2 and P-3 groups is not important but that of the P-4 group significantly increased over the other groups. This latter observation indicates that the "adrenarche" itself does not influence the pregnenediol output exceeding the general growth tendency. The increase in the pregnenediol output is probably a consequence of the elevated ovarian progesterone secretion due to the start of the ovarian cycle.

The values of adrenal androgens reflect the progress of "adrenarche". The increase in androgen production characteristic of "adrenarche" has begun already in the 7-9-year group and continues in puberty. The androsterone + ethiocholanolone (DHA ratio, as well as the pregnanediol/pregnenediol values provide valuable information concerning the metabolism of Δ5-3β-hydroxy steroids and also for the activity of Δ5-3β-hydroxy steroid dehydrogenase in the body. The ratio was calculated individually, from the steroid values of the children; in the case of pregnanediol our recently published data (Kecskés et al., 1982) were also considered. The mean, S.D. and limit values of the age groups are depicted in Table 4. In the 2-6-year group the 3α-hydroxy/Δ5-3β-hydroxy ratio with the C_{19} steroids is five times higher than with the C_{21}, steroids while this difference is tenfold in the 7-9-year and 10-14-year groups. These results clearly demonstrate that DHA, having an androgene character, is much more intensively metabolized at its C-3, than the steroid intermediate pregnenolone and its metabolite, pregnenediol.

DISCUSSION

Based on abundant physiological and clinical experience, urinary pregnanediol excretion is considered as a firm index of

Table 4. Ratio of pregnanediol/pregnenediol (3α-OH/$\Delta5$-3β-OH-C_{21}) and androsterone + etiocholanolone/DHA (3α-OH/$\Delta5$-3β-OH-C_{19}) by healthy girls of different ages

Age (years)	n	3α-OH/$\Delta5$-3β-OH-C_{21} mean ± SD	Range	P	3α-OH/$\Delta5$-3β-OH-C_{19} mean ± SD	Range	P	
2 - 6	11	1.03 ± 0.89	0.29 - 2.7	n.s.	4.68 ± 4.04	1.20 - 12.0	n.s.	<0.01
7 - 9	9	0.59 ± 0.35	0.24 - 1.34	n.s.	10.11 ± 8.47	2.30 - 28.0	n.s.	
10 - 14	13	0.97 ± 0.94	0.24 - 3.55		12.98 ± 5.94	2.50 - 22.1		

progesterone production notwithstanding the fact that pregnenolone is also an important precursor of pregnanediol. At low pregnanediol values pregnenolone may become a more abundant precursor of pregnanediol than progesterone (Romanoff et al., 1966). The relationship through progesterone secretion, between pregnanediol and corpus luteum is well known. A small amount of progesterone is secreted by the ovarium in the follicular phase, as well (Abraham and Chakmakjian, 1973). Of the numerous steroid determinations from human adrenal venous blood only Short (1960) reported data on progesterone secretion. It has also been supported by indirect experiments, demonstrating a marked increase in progesteron level in the periferial venous blood after the administration of ACTH (Bermudez and Lipsett, 1972).

The other pregnanediol precursor, pregnenolone is also secernated by both the adrenal (Certel et al., 1963) and the ovarium (Abraham and Chakmakjian, 1973). In the follicular phase the plasma level of pregnenolone exceeds threefold that of progesterone.

In children, the level of both progesterone and pregnenolone is a fraction of that in adults. In puberty, the elevation of pregnenolone level is significant only following the 10th-year, while for progesterone this occurs only after 12-years of age as compared to the 7-year group and it approaches the value of adults at the and of puberty (Apter and Vihko, 1977).

Few data are available concerning pregnanediol excretion in childhood. In 48 girls aged 3-15-years this value was found to be 0.72±0.62 (Bergstrand and Gemzell, 1957). The authors found no correlation between age and pregnanediol excretion and, with 3 exceptions, no difference was found between girls in pre-puberty and puberty, as well as girls in puberty and those already having regular periods. In the three exception all cases the patients collected their urine before the expected period and the pregnanediol value indicated the presence of corpus luteum. The above authors found the range of pregnanediol excretion in prepubertal and pubertal girls identical to that of males.

We observed that pregnanediol excretion was low in prepuberty, and has increased significantly during puberty in correlation with body growth until the 4th phase of puberty. Pregnanediol excretion per kilogramm body weight increased only in the 4th phase of puberty. These girls had already had periods but, on the basis of pregnanediol value, the cycle was still monophasic. The mean pregnanediol value of this group was similar to that of adult males reported earlier (Juricskay and Kecskés, 1978). The discrepancy between our results and those of Bergstrand and Gemzell (1957) can be explained by methodological differences. For the quantitative determination of pregnanediol these authors used the column chromatographic method of Klopper et al. (1955) followed by the photometric determination of the sulfuric acid chromogen of pregnanediol. The bacground value of this determination, however, is much higher than that of the gas-chromatographic method. Among other authors using photometric methods Talbot et al. (1952) could find pregnanediol in the urine only under pathologic circumstances, while Balassi and Ricca (1951) detected only traces of pregnanediol before 5-years of age.

Comparing the pregnanediol excretion values (2-6-year: 2.63±1.72; 7-9-year: 2.48±1.16 µg/kg/day) with the pregnanediol levels of the same children (3.48±1.8; and 6.4±5.4, resp., Kecskés et al., 1982) the ratio of the two (pregnenediol/pregnanediol) is 1.5-2.5. The pregnenolone/progesterone ratio in the plasma of girls of similar age was found by Apter and Vihke (1977) to be 3-6, therefore we may conclude that pregnenolone is the precursor of a considerable fraction of pregnenediol. In this transformation, taking place in non-endocrine organs, oxidative, reductive and isomerisation steps follow each other, catalysed by Δ^5-3β-hydroxy-(C_{21})-steroid dehydrogenase, $\Delta^{4,5}$-isomerase, Δ^4-β-reductase and 3α-hydroxy-steroid dehydrogenase. The enzyme Δ^5-3β-hydroxy-(C_{19})-steroid dehydrogenase and the already mentioned other enzymes perform the transformation of DHA to ethiocholanon and Δ^4-α-reductase convertes DHA to androsterone. Presumably the rate limiting enzyme is the

Δ^5-3β-HSD of the liver. This is supported by the relative increase of the urinary excretion of DHA, compared to other metabolites, following the administration of DHA (Kirschner et al., 1963). The enzymes, carrying out the transformation are most abundant in the liver, although they can be detected in much lesser amounts in other organs (skin, lung) as well. Only few data are available regarding the <u>in vivo</u> behavior of Δ^5-3β-HSD. Fehér (1966) measured the DHA/ethiocholane ratio in 14 girls and it was found to be 0.128. The inverse of this value (7.8) is similar to the ratios of 3α-OH/Δ5-3β-OH-C_{19} obtained by us (Table 4). Our comparative studies indicate that Δ^5-3β-hydroxy-(C_{19})-steroid dehydrogenase activity in childhood is 5-10 fold higher than that of Δ^5-3β-hydroxy-(C_{21})-steroid dehydrogenase.

Our conclusions are in accord with the results of Björkhem et al. (1972). These authors have found that the microsomal fraction, prepared from liver biopsies obtained by surgery in male and female patients, converted DHA to androstenedione up to 55-60 per cent in the presence of NAD, while the conversion of pregnenolone to progesterone was a mere 4-6 per cent. The differences found in the 3α-hydroxy/Δ^5-3β-hydroxy ratio can also be determined by the liver enzymes.

Δ^5-3β-HSD of bovine adrenal transforms DHA to androstenedione and pregnenolone to progesterone at approximately the same rate. The two substrates are competitive antagonists of each other and, as proved also by kinetic measurements, both transformations are carried out by the same enzyme (Neville et al., 1969). Similar results were reported for human adrenal, too (Yates and Desphande, 1975). Δ^5-3β-HSD is regarded as a microsomal enzyme similarly to Δ4,5-isomerase. The two microsomal enzymes could not be separated from each other during purification (Ford and Engel, 1974). Δ4,5-isomerase converts the C_{19} and C_{21} substrates on the same position (Cheatum et al., 1967), furthermore its specific activity is manyfold as compared to that of Δ5-3β-HSD. Thus the rate limiting enzyme of the transformation of Δ5-3β-hydroxy-steroids to Δ4-3-ketosteroids is Δ5-3β-HSD (Neville et al., 1969).

To explain the low pregnanediol/pregnenediol ratio found by us as well as the low conversion of pregnanolone to progesterone observed by Björkhem et al. (1972), we assume that the liver Δ5-3β-HSD is an allotype of the adrenal enzyme and the relative activity of the former toward pregnenolone substrate is less. The allotype hypothesis has been put forward by Eastman and Neville (1977) for pathological adrenal tissue where the Δ5-3β-HSD activity was lost only towards a Δ5-3β-hydroxy-steroid. They suggest that it is a minor change in a structural gene, or some alteration in protein synthesis, or in the cellular environment that may result in a variant in which the multiple substrate specificity of the enzyme is limited.

Besides the allotypes the influence of substrates, manifested by competition, may also play a role. However, we do not think, this to be the case since the ratio of pregnenolone (Apter and Vihko, 1977) and the free DHA (Hopper and Yen, 1975) in the plasma at the age of 7-8y is 1:1, and in puberty it is 1:2. Competition may also take place between the conjugating enzymes of the liver (primarily UDP-glucuronyl transferase) and Δ5-3β-HSD. This is indicated by Björkhem and Kalmar (1975) who investigated the 3α-^{3}H-pregnenolone metabolism <u>in vivo</u> using bile fistula. They found that, parallel to Δ5-3β-HSD, ^{3}H-pregnenolone metabolism was also affected by the conjugating enzymes of the liver. Conjugation prevented the oxidation at C-3 and most of the radioactivity in the bile was associated with the glucuronide fraction.

We suggest that the low pregnanediol/pregnenediol ratio, irrespective of any biochemical mechanism is significant biologically. We believe that, due to this phenomenon the probability of a temporal high pregnenolone leakage (for -xample from the andrenals by stress), resulting in an elevated progesterone level in the plasma that could disturb the characteristically sensitive regulation of the hypothalamus-hypophysis-gonad system in the female body, will be diminished.

REFERENCES

Abraham, G.E., Chakmakjian, Z.H. (1973) J. Clin. Endocr. 37, 581

Albright, F., Smith, P.H., Fraser, R. (1942) Amer. J. Med. Sci. 204, 625

Apter, D., Vihko, R. (1977) J. Clin. Endocr. 45, 1039

Balassi, G.P., Ricca, C. (1951) Minerva Ginec. 3, 277

Bergstrand, C.G., Gemzell, C.A. (1957) J. Clin. Endocr. 17, 870

Bermudez, J.A., Lipsett, M.B. (1972) J. Clin. Endocr. 34, 241

Björkhem, I., Einarsson, K., Gustafsson, J.A., Somell, A. (1972) Acta Endocr. 71, 569

Björkhem, I., Karlmar, K.E. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 1835

Cheatum, S.G., Douville, A.W., Warren, J.C. (1967) Biochim. Biophys. Acta 137, 172

Dohm, G. (1973) Beitr. Pathol. 150, 357

Eastman, A.R., Neville, A.M. (1977) J. Endocr. 72, 225

Fehér, T. (1966) Clin. Chim. Acta 14, 91

Ford, H.C., Engel, L.L. (1974) J. Biol. Chem. 249, 1363

Hopper, B.R., Yen, S.S.C. (1975) J. Clin. Endocr. 40, 458

Juricskay, Zs., Kecskés, L. (1978) Acta Biochim. Biophys. Acad. Sci. Hung. 13, 279

Kecskés, L. (1982) Acta Biochim. Biophys. Acad. Sci. Hung. 17, 46

Kecskés, L., Juricskay, Zs. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. 10, 37

Kecskés, L., Juricskay, Zs., Czira, G. (1980) Acta Biochim. Biophys. Acad. Sci. Hung. 15, 49

Kecskés, L., Juricskay, Zs., Kosztolányi, Gy., Szécsényi, M. (1981) Acta Biochim. Biophys. Acad. Sci. Hung. 16, 57

Kecskés, L., Juricskay, Zs., Tatai, Zs., Szécsényi, M. (1982) Acta Paediatr. Acad. Sci. Hung. 23, 151

Kirschner, M.A., Lipsett, M.B., Wilson, H. (1963) Acta Endocr. 43, 387

Klopper, A., Michie, E.A., Brown, J.B. (1955) J. Endocr. 12, 209

Neville, A.M., Orr, J.G., Engel, L.L. (1969) J. Endocr. 43, 599

Oertel, G.W., Eberhard, K., Zimmermann, W. (1963) Hoppe-Seyler's Z. Physiol. Chem. 331, 77

Romanoff, L.P., Grace, M.P., Baxter, M.N., Pincus, G. (1966) J. Clin. Endocr. 26, 1023

Short, R.V. (1960) Biochem. Soc. Symposia 18, 59

Talbot, N.B., Sobel, E.H., McArthur, J.W., Crawford, J.D. (1952) in Functional Endocrinology from Birth through Adolescence, Harward University Press

Yates, J., Desphande, N. (1975) J. Endocr. 64, 195

Acta Biochim. Biophys. Hung. 21/1-2/, pp. 81-97 /1986/

THE PLASMA MEMBRANE AS RADIOSENSITIVE TARGET*

Gy.J. Köteles

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary

(Received August 4, 1984)

SUMMARY

The rapidly accumulating knowledge on the structure and function of cell membranes stimulates the relevant radiobiological studies. Several factors rendering the membrane system a radiosensitive target as well as radiation-induced alterations of plasma membrane are reviewed. For understanding subcellular effects the need of projecting physical energy deposition events over the biological scenary in respective volumes is emphasized. The possible role of membrane alterations in the fate of irradiated cell is discussed.

INTRODUCTION

The biological effects of ionizing radiations on cell membranes has attracted attention since the early days of radiobiology. The increase of permeability for salt and sugar of skin and heart tissue was observed in the twenties and it was postulated that the effect is related to change of charge at cell membranes (Kovács, 1928). In accordance with the actual interest and level of cell biology during the various periods, radiobiological studies have been performed and data from large number of references mainly on radiochemical reactions, fatty acids, SH-containing proteins and other biochemical effects have been summarized in several reviews (Myers, 1970; Wallach, 1974; Patrick, 1977). The possible role of membrane damage was taken as basis of the "enzyme-release" theory as an explanation of radiation-induced cell death by degradative enzymes released through damaged lysosomal membranes (Bacq, Alexander, 1961).

*Invited paper read at the 12th Meeting of Hungarian Biophysical Society, Budapest

In the early seventies, new experimental data and theories lead to a dynamic image of the structure and function of plasma membranes (Singer, Nicolson, 1972; Nicolson, 1976; Israelachvili, 1977). These put again radiobiological studies on the membrane system into new perspectives (Köteles, 1979; 1982). The rapidly accumulating data can not be reviewed here, only a few milestones are given in Table 1 outlining certain phases of research when new aspects have been arisen in the literature. Therefore, in the present paper only some conditions and experimental evidences are shown which point to the sensitive reactions of membranes upon ionizing radiations rendering them either a primary target or a contributory one.

Components and Conditions Rendering the Plasma Membrane Susceptible for Ionizing Radiations

In the era of high resolution electron microscopy it has become quite obvious that the membrane system including the plasma membrane, membranes of organelles like nuclei, mitochondria, the lysosomes, the endoplasmic reticulum represents a considerable volume for energy absorption. In fact, it would be interesting to have a good assessment on the proportion of this volume as compared to that of the whole cell. These structures are built up by molecular complexes of lipids, proteins and carbohydrates. Among the lipids the polyunsaturated fatty acids (PUFA) are the most sensitive molecules, since through lipoxigenation they degrade with such high G-value, i.e. number of altered molecules per 100 eV energy absorption (Mead, 1952; Strazhevskaya, Struchkov, 1977; Köteles, 1979) which suggests a chain reaction (Petkau, 1980). The degradative products are of malondialdehyde type (Konings et al., 1979). Due to the interaction of inorganic and organic free radicals a chain reaction might be set on leading to a long-lasting and gradual decomposition of membranes (Petkau, 1980; Köteles, Kubasova, 1982). In the light of recent investigations, however, the PUFAs built into membrane structure proved to be less radiosensitive than in solution or in micellar or liposomal structures,probably because of the

TABLE 1 List of reviews and articles pointing to various aspects of radiation effects on membranes

Effect/Phenomenon	Reference	
Permeability for ions	Kovács	1928
Autoxidation of fatty acids	Mead	1952
Enzyme-release theory for cell death	Bacq, Alexander	1961
Various biochemical effects	Myers Wallach Patrick	1970 1974 1977
Membrane as target in presence of oxygen	Alper	1971
Perturbation of membrane structure and consequently function	Köteles	1979
Contributions of membrane damage to sensitivity and repair of other targets	Szumiel	1981
Correlation of microstructural and functional alterations, biological consequences of membrane damage	Köteles Edwards et al.	1982 1984

natural antioxidant activity of cells (Konings, Drijver, 1979). It was also demonstrated that, as far as cell survival is concerned, cells with plasma membranes enriched in PUFAs were not more sensitive than those with original composition (Wolters, Konings, 1982). Our own experiments, too, pointed out that only approximately 30 per cent of arachidonic acid (one of the most vulnerable PUFA, 20:4) degraded rapidly in human lymphocytes irradiated in vitro (Kubasova et al., 1984). Further investigations are needed to clear up the structural sensitivity of phospholipid molecules as it has been suggested that rather low energies might cause broken ("gauche") configurations (Györgyi, 1980) leading to disturbed lipid bilayer or lipid-intramembrane particle contacts. The relationship of the factors reducing radical effects like natural antioxidants,

radical eliminating enzymes and their availabilities or localizations in or near the membrane structure have to be studied.

Much less is known of the possible structural alterations of protein components. In contrast to the radiosensitive lipids, the protein structure and function, like enzyme activity, can be altered in vitro only by doses of several orders larger magnitude than that effective in vivo. Therefore, it is intriguing to think that alterations of enzyme activities - either increases or decreases as both of them occur even in the case of the same enzyme, depending on dose - in irradiated cells are caused by the altered intimate intermolecular relations between proteins and lipids (Köteles, 1979). This is a further condition which might contribute to functional impairment of membranes.

The spatial arrangement of various membrane components is ensured by hydrophilic and hydrophobic forces of rather low energies, i.e. less than 1 eV. The breaking of these weak bonds by energy deposition events may result in disconnections of certain multimolecular complexes of structural or functional importance. Good examples are the "signal-clusters" consisting of receptors and information processing enzymes like that described for concanavalin A receptor-Na^+K^+-dependent ATPase-lysolecithintransferase system, the close functional and spatial relationship of which is an essential condition for the activation of lymphocyte by mitogen lectin (Szamel et al. 1982). The biophysics of dynamics of surface binding sites, the mobility of proteins providing such constellations are currently studied by fluorescence energy transfer technique (Damjanovich et al., 1982). Further examples of multimolecular complexes are the alternating domains differing in microviscosity, the regions ensuring and maintaining various receptor requirements (Köteles, et al., 1983).

Last but not least, the possibility can be raised that derangement of plasma membrane might originate from damaged intracellular structures like microfilament network playing role in anchoring receptors (Nicolson, 1976) or from altered processes like those providing energy for biological functions (Bacq, Alexander, 1961).

Radiation-Induced Alterations of Plasma Membrane

An immense literature is available either on isolated membranes or on those of various cells irradiated in wide dose-ranges and with various sensitivities. In accordance with one of the present trend of contemporary radiation biology those effects are of main concern which are provoked by relatively low doses, i.e. causing sublethal or even nonlethal damages to the cells*. In the previous chapter it was suggested that those functions are expected to be highly sensitive which are based on the integrity of multimolecular arrangements. The following examples (mostly from our laboratory) illustrate a few of such effects, i.e. effects exerted on surface charges, on binding of protein molecules and virus particles, and on regenerative processes.

The surface charges of plasma membranes play decisive roles in any contact and reaction of the cell. The normal surplus of negatively charged molecules on the outer surface changes during physiological processes like mitogen stimulation, phagocytosis, endocytosis. It has been reported that ionizing radiations cause alterations in surface charges as tested by cell microelectrophoresis. Controversial data came, however, mostly due to the cell types, timing and the rather high doses applied (for refs. Köteles, 1979). Obviously, minute changes can not be manifested in mobility alterations of the whole mass of cell. Therefore, it seemed to be promising to apply a more sensitive technique, namely the binding of charged ligands. By this way it was expected to detect regional differences, too. We have studied the binding of cationized ferritin (CF) by transmission electron microscopy (Köteles et al., 1983). It was found that the amount of CF bound to the surface of human fibroblasts decreased within 10 minutes following non-lethal doses of x-irradiation. The original binding conditions recovered within an hour.

*Sublethal damage: the accumulation of which may result in cell lethality, non-lethal damage that does not lead to cell death (ICRU, 1979).

The observations suggested two types of changes: first, the decrease of binding at the existing binding sites, and second, the reduction of the number of binding sites, i.e. the surface coverage of cells.

It is interesting to mention that the character and direction of alterations of surface charges and of transmembrane potentials depend on radiation quality and dose rate. We have observed that tritium beta irradiation results not only in a decrease of negative charges but even in a shift to positively charged surface areas (Köteles, et al., 1983). Other authors demonstrated for transmembrane potential of lung cells that while gamma-irradiation provoked a sequential oscillatory de- and hyperpolarization, alpha-irradiation resulted only in a depolarization (Steinhäusler et al., 1983). No detailed studies are available on the mechanism of these phenomena. For the loss of negative surface charges the "sinking" of carrier molecules, mostly sialic acids, into a deeper region of erythrocyte membrane was suggested (Sato et al., 1977). Immediate desialylation in gamma-irradiated mouse lymphocytes was also reported, although only following lethal doses (Banchereau et al. 1982).

The binding capabilities of cells for various ligands change sensitively following irradiation. This was demonstrated by studies with ligands having different "receptor-requirements" The amount of concanavalin A bound to irradiated fibroblasts, platelets, lymphocytes, erythrocytes of murine and human origin increased early and temporarily (Köteles et al., 1976; Kubasova et al., 1981 a,b; 1982). The binding of CF to fibroblasts decreased rather early, too, as mentioned above. The attachement and penetration of poliovirus to the proper host cell was facilitated by x-irradiation (Köteles, Kubasova, 1982; Köteles et al., 1983). It was, however, noted that the rearrangement of original membrane conditions took longer time for these protein ligands than for the return of normal appearance of surface charges. This suggests that for the protein ligands complex binding area is required, i.e. the binding site includes not only charged groups but also a certain multimolecular constellation.

By binding studies with proteins and virus particles we have presented evidence that radiation effects on plasma membranes depend on the radiation dose, cell type, physiological state like age, the functional perturbation of membrane appears early, it is reversible and proved to be rather general phenomenon as it was found to be valid for various mammalian cells.

Scanning electron microscopic analyses also demonstrated well-defined surface alterations already 10 minutes after x-irradiation above 0.25 Gy (Somosy et al., 1983). These include the appearances of filopodia and lamellipodia as well as plasma bridges connecting the detached membranes with the substrate. Initial signs of surface restoration could be observed 4 hours after irradiation while the full restoration of the confluent monolayer was completed at 24 hours.

The derangement of membrane depend also on radiation quality and dose-rate. In contrast to the data obtained after x-irradiation, tritium beta irradiation of cells resulted in alterations qualitatively different from x-irradiation as detected by binding studies and micromorphological appearances of cell surfaces (Köteles et al., 1983). Long- and short-term treatments of cells with ^{3}H-thymidine and ^{3}H-water in the concentration range from 3,7 kBq (0,1 μCi) per ml induced repressed bindings of concanavalin A, CF and poliovirus, i.e. ligands having various receptor requirements. Scanning electron microscopic pictures showed a flattening of cells and smoothening of their surfaces. The phenomena also proved to be reversible.

As far as the regeneration of plasma membrane of irradiated cells is concerned, this seems to be the largest white spot at the moment. From the data mentioned it might be concluded that the various features and functions are not restored with the same speed. Possibly, the regeneration involving new syntheses of membrane components or new assembly of multimolecular complexes need more time than the re--appearence of temporarily masked surface charges. This is supported by our findings that the full replacement of

^{14}C-arachidonic acid is a rather protracted process lasting for several hours (Kubasova et al., 1984). The replacement of large units like damaged membrane regions might be also delayed by the damages of prefabricated intracellular membrane elements and of their assembly hall, the Golgi complex (Kubasova et al., 1976). The protracted regeneration and the oscillatory nature of surface phenomena might be due to the long-lasting existence of organic radicals in the cells inhibiting the restoration of normal membrane conditions.

Energy Deposition in Cellular Microstructures

The interaction of electromagnetic quanta or particles with cells or intracellular organelles and biomolecules is of stochastic nature. For studies of biological reactions within subcellular structures like membranes in function of absorbed dose the classical terms of dosimetry, - i.e. - energy absorption in a volume much larger than the cell - can not be used to express dose-effect relationship due to the great inhomogeneity of energy deposition. The effects also depend on radiation quality as the densely and sparsely ionizing radiations, i.e. those of the high and low LET values hit different numbers of cellular of intracellular targets. This is illustrated by an example as follows (Rossi, 1979): Irradiation of a cell of 5 μm diameter with 10 mGy (1 rad) by 1 MeV gamma-rays induces the formation of appr. 150 ion-pairs while with 1 MeV neutrons it produces appr. 6500 ion pairs along the path of the particle, i.e. on its "track". In additions, while the same effect can be expected in all cells of gamma-irradiated tissue, only about 2 per cent of cells are involved in neutron-irradiated tissue.

Thus, in the recent years intensive research has begun to estimate the energy distribution from electromagnetic and particle radiations in small volumes, usually smaller than the nuclei of mammalian cells (Rossi, 1979; Goodhead, 1982). This research area is called "microdosimetry". By the use of its considerations and tools either including physical measurements by Rossi-counter or assessments based upon Monte Carlo

calculations it might be accomplished for radiation biology of the future to project the possible physical events over the biological structure investigated and by this way to compare the energy deposition with energy contents maintaining the respective biological and chemical structure, the composition and biological function within volumes of micro- or nanometer ranges. In other words, having the information on energy distribution from microdosimetric measurements and calculations in terms of "specific energy*", it has to be analysed whether in a certain target volume the forces sustaining the molecular and supramolecular functional units as well as the integrity of individual molecular components can be overcome by a few or even a single energy deposition event. The effects and perhaps the detectability, of course, depend on the specific functions and features of the molecules and functional units within the investigated volume. Therefore, both the data banks of the physical events and the structural-functional information for the biological volumes should be superposed to have an idea of possibility and probability of interactions of radiation and biological material in ranges from micrometer down to nanometer extensions. This relationship for membranes is illustrated in Fig. 1.

Recently, considerable information has been collected for the slowing down spectra of electrons of appr. 100 keV down to 1 keV and 10 eV. This approach is very important because even the energy of gamma-radiation is decomposed through such electrons, i.e. appr. 34 per cent by less than 5 keV and 26 per cent by less than 1 keV electrons (Goodhead, 1982). Further, the analysis of the fate of low energy electrons helps to understand the biological effects of low energy beta irradiation, too, like that of ^{3}H and ^{14}C. Studies on the decomposition of radiation field by reactions of electrons from ultrasoft x-rays revealed that within a path-length of a few nano-

*Specific energy is the quotient of e/m where e is the energy imparted by ionizing radiation in a volume element with a mass m (ICRU, 1979).

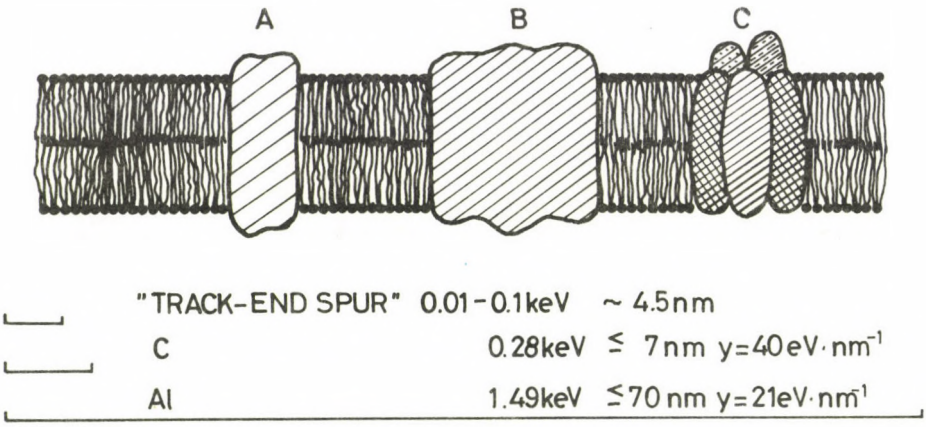


FIG. 1 Examples for dimensions of plasma membrane domains and path-lengths of low energy electrons. The particles are the "track end" photo-and Auger-electrons produced from carbon (C) and aluminium (Al) ultrasoft x-rays.
The integrated proteins illustrate the dimensions of glycophorin in erythrocyte or small intramembrane particle (IMP) in lymphocytes (A), large IMP in lymphocytes (B), acetylcholine receptor (C). The "y" value is the lineal energy.

meters considerable number of energy deposition events occur transferring 10-40 eV per nanometer distances (Goodhead, Brenner, 1983). Similarly, at the end of a ^{3}H beta particle track more than 20 eV per nanometer is transferred.

Another example is the calculation on the decomposition of a 5 keV electron in water (Turner et al., 1980). It has been pointed out that a single electron in a path not longer than 700 nm produced 385 inelastic events at 10^{-15}s from the initial energy deposition and the total number of various species of radicals was 1196 over the complete track at 10^{-11}s. The distribution of radicals showed for instance that in a sphere of 10 nm radius 6-61 hydroxyl radicals ($OH^{\bullet}$) were present. Later, the distribution of radicals proved to be diffusion-controlled, though these did not cover longer distance than a few further nanometers either. Concerning the membrane region, a detailed analysis is needed on the forces keeping the structural-functional conditions in operation. When lineal energy transfer* in the range of 10-50 eV per nm

*Lineal energy is the energy imparted by individual charged particles in a volume of a specified size (ICRU, 1979).

occurs, it seems to be enough to break even several covalent bonds of appr. energy 3 eV and certainly enough to disrupt large number of secondary intermolecular bonds of much smaller energy content - 0,01-0,5 eV - like van der Waals or hydrogen bonds. The latter plays role in ligand-receptor interaction as well as in keeping the integrated proteins in their lipid milieu. These considerations forecast the era when the sensitivities of even certain domains of membranes can be assessed or when the consequence of a single energy deposition event in a certain volume can be predicted. The importance of such thoughts is supported by the dynamic reactivity of membranes, e.g. a binding event at a proper domain, the receptor capping or mitosis. Thus, poking a membrane area may influence the re-arrangement of the whole, resulting in multi-fold reactions.

Certainly, many observations, including our studies on biological effects of tritium beta irradiation on cell surface alterations and membrane derangement, can not be explained by "macrodosimetric" terms, as the doses delivered from rather low concentrations are very low, e.g. for 37 kBq (1 μCi) per ml ^3H_2O the calculated dose-rata is appr. 0,1 mGy per hour (Feinendegen et al., 1971). Data from microdosimetric calculations of this low-concentration effect, as the final outcome from a 5 keV electron track (i.e. similar to the tritium), show a large number of radicals with an overwhelming positive net charge (Turner et al., 1980). This rather unusual condition might maintain a surplus of neutral or positive charges on the cell surfaces. So, even if this happens at certain domains, as predicted from the extension of the track structure and observed in our experiments, it can have biological effects altering an area where units of receptor and signal-transferring system are located. Co-operative processes in membranes, however, like fluidity alterations, receptor unit interactions might modify radiation effects by extending them from local events to larger areas. It is also conceivable that different membrane effects are provoked at different areas due to the different radiosensitivities of certain domains, like

those rich in PUFAs or lipid-protein or enzyme transition phase, sensitive centres of integrated enzymes. Thus, further progress can be expected in the correlation between very low doses, perhaps a few energy deposition events and possible biological effects. The discrepancy, however, between the time scale of primary and secondary events and the chemical, biochemical, structural processes point to many still unknown factors. Physical and biological processes have to be correlated. On the one hand are the ways and means of energy deposition like track structure or non-localized transfers of energy in collective oscillations or "plasmons" (Berger, 1980) which may lead to "shaking up" membrane domains. On the other hand, the vulnerability of domains, size of domains, their biochemical composition, nature and densities of various inter- and intramolecular bonds and forces have to be considered. Membrane structure and membrane phenomena seem to be advantageous biological models for such studies, too.

Role of Membrane Alterations in the Fate of Irradiated Cell

The radiosensitive target can be defined as a structural--functional part of volume of the cell, the radiation-induced alterations of which result in abnormal changes or damages of cellular functions and integrity. This principle presumes that this volume embodies molecules or their complexes of vital importance. The dose-effect relationships are usually analysed for permanent "end-points" like cell death, survival rate, chromosome aberrations, gene mutations malignant transformation. These are mostly irreversible conditions which develop as results of unrepaired or misrepaired damages despite the enormous efforts of cells for regeneration. It is obvious that the lesions of primary importance in all of these cases lay in the DNA. Upon irradiation, however, not only the chromatin substance is affected upon but many other parts, volumes, molecular complexes or organelles of the cell. Radiation effects are, of course, stochastic and the regeneration of extra-chromatin targets, the consequences of these injuries or failures in their regeneration are mostly unknown. It is also

obvious that during the regenerative processes many temporary alterations have to be present in the cell which either develop into permanent endpoints or influence the efficiency of rapair and regeneration. It is reasonable to think that cell membranes play central role in both directions, i.e. they influence the development of any radiation effect and they influence their reparation. As a consequence, they take part in determining the fate of the cell. The relations of developments of temporary and permanent effects are illustrated in Table 2.

In the forerunning chapters it was pointed out that the features of the irradiated membranes represent conditions which might lead to deterioration of its own function. Further important problems are whether the membrane alterations can influence extra-membraneous radiation effects like metabolic or structural alterations, for instance radiation-induced repair, mitotic death, chromosome aberrations, mutations, malignant transformations. The correlations of membraneous and extra-membraneous effects are hardly known. In the following paragraphs a few possibilities are raised just to call attention to some important research trends of the future.

Membrane perturbation may affect such functions as recognition of regulatory ligands, permeability for metabolic precursors and for elimination of degradation products. Irradiated membranes themselves are sources of degradative products which might be either mutagenic or might represent further organic radicals. Failures in providing the necessary metabolites for repair and regeneration can also be attributed to membrane damages influencing other processes. Misrecognition of virus particles or infections by cellular parasites may cause longterm, late consequences. Membrane specific drugs, chemicals, as well as hyperthermia also have to be considered when combined effects of radiation are studied.

The fate of the cell depends on the physical conditions of irradiation and on the biological sensitivity. Both of them involve many still unknown factors. According to our

TABLE 2 Phases, targets, temporary and "end-point" effects in irradiated cells

Phase	Physical	Chemical	Biochemical	Biological
Time-range	10^{-18} - 10^{-12} s	10^{-12} - 1 s	1 s - minutes	minutes - days
Events	Primary energy deposition - ionization - excitation - vibration	Secondary energy deposition - radical reactions		
	Direct damage to biomolecules	Indirect damage to biomolecules		
Effects	Levels: / Main targets: - molecular: DNA, phospholipids, proteins - supramolecular: multimolecular complexes (nucleic acids - proteins - lipids) - like chromatin membrane domains		Temporary - single and double stranded breaks of DNA - change of superhelical conformation of nucleosomes - perturbation of membranes - structural - functional - metabolic including disturbances of co-ordination of concomitant repair and regenerative processes	Final, "end-points" - full regeneration - mutations - chromosome aberrations - malignant transformations - cell death - reproductive - interphase

present view, the membranes can be considered either as targets of primary importance if their lesions lead directly to cell damage (repairable or not), or as co-operative targets modifying the consequences of extramembraneous damages by their temporary perturbances. The membranes as radiosensitive targets become of considerable importance not only at large radiation doses leading to interphase death of cells but also when the biological effects of sublethal or non-lethal doses are studied.

I do hope that the distinguished members of the Hungarian Biophysical Society could get a concise impression on a few aspects of membrane radiation biology as they are at present, as well as on the possibilities in research applying ionizing radiation either as a tool by cell biologists or the main subject of studies in the hands of radiation biologists for the sake of the exploitation of its power or for the protection against its harmful effects.

Acknowledgements

I am indebted to Professor J. Tigyi, Academician, President of the Hungarian Biophysical Society and Professor Gy. Rontó, Secretary General of the Society for their invitation to deliver this lecture, to Professor L.B. Sztanyik, Director General of the "Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene for his continuing interest and support of this work, to my co-workers Drs. T. Kubasova and Z. Somosy.

REFERENCES

Alper, T. (1971) In: "Biophysical aspects of radiation quality", IAEA, Vienna, pp. 171-183

Bacq, Z.M., Alexander, P. (1961) Fundamentals of Radiobiology, Pergamon Press, Oxford

Banchereau, J., Dubos, M., Agneray, J., Drouet, J. (1982) Biochem. Biophys. Res. Comm. 104, 512-516

Berger, M.J. (1980) Proc. 7th Symp. Microdosimetry, eds. Booz, J., Ebert, H.G., Hartfiel, H.D., Hartwood Ac. Publ. pp. 521-534

Damjanovich, S., Trón, L., Szőllősi, J. (1982) MTA Biol. Oszt. Közl. 25, 495-504

Edwards, J.C., Chapman, D., Cramp, W.A., Yatvin, M.B. (1984) Prof. Biophys. Molec. Biol. 43, 71-93

Feinendegen, L.E., Ertl, H.H., Bond, V.P. (1971) In: "Biophysical aspects of radiation quality", IAEA, Vienna, pp. 419-429

Goodhead, D.T. (1982) Radiat. Res. 91, 45-76

Goodhead, D.T., Brenner, D.J. (1983) Phys. Med. Biol, 28, 485-492

Györgyi, S. (1980) MTA Biol. Oszt. Közl. 23, 379-388

ICRU (1979) Quantitative concepts and dosimetry in radiobiology. International Commission on Radiation Units and Measurements, Report 30

Israelachvili, J.N. (1977) Biochim. Biophys. Acta 469, 221-225

Konings, A.W.T., Drijver, E.B. (1979) Radiat. Res. 80, 494-501

Konings, A.W.T., Damen, J., Trieling, W.B. (1979) Int. J. Radiat. Biol.

Kovács, K. (1928) Strahlentherapie 30, 77-85

Köteles, G.J., Kubasova, T., Varga, L. (1976) Nature 259, 507-508

Köteles, G.J. (1979) Atomic Energy Rev. 17, 3-30

Köteles, G.J. (1982) Radiat. Environm. Biophys. 21, 1-18

Köteles, G.J., Kubászova, T. (1982) MTA Biol. Oszt. Közl. 25, 41-58

Köteles, G.J., Kubasova, T., Somosy, Z., Horváth, L. (1983) In: "Biological effects of low-level radiation" IAEA, Vienna, pp. 115-128

Kubasova, T., Varga L., Köteles, G.J. (1976) Int. J. Radiat. Biol. 29, 533-540

Kubasova, T., Varga, L.P., Köteles, G.J. (1981a) Int. J. Radiat. Biol. 40, 175-186

Kubasova, T., Köteles, G.J., Varga, L.P. (1981b) Int. J. Radiat. Biol. 40, 187-194

Kubasova, T., Köteles, G.J., Varga, L.P. (1982) In: "Cell membrane probes as biological indicators in radiation accidents", IAEA, Vienna, pp. 113-124

Kubasova, T., Szamel, M., Somosy, J., Köteles, G.J. (1984) Acta Biochim. Biophys. Acad. Sci. Hung. (in preparation)

Mead, J.F. (1952) Science 115, 470-472

Myers, D.K. (1970) Adv. Biol. Med. Phys. 13, 219-233

Nicolson, G.L. (1976) Biochim. Biophys. Acta 457, 57-108

Patrick, G. (1977) In: "Mammalian cell membranes" eds. Jamienson, G.G., Robinson, D.M., Vol. 5., p. 72. Butterworth, London

Petkau, A. (1980) Acta Physiol. Scand. Suppl. 492, pp. 81-90

Rossi, H.H. (1979) Radiat. Environ. Biophys. 17, 29-40

Sato, C., Kojima, K., Nishizawa, K. (1977) Radiat. Res. 69, 367-374

Singer, S.J., Nicolson, G.L. (1972) Science 175, 720-731

Somosy, Z., Kubasova, T., Köteles, G.J. (1983) J. Radiat. Res. 24, 109-117

Steinhäusler, F., Reubel, B., Heidegger, W., Huber, M., Pohl.Rühling, J. (1983) In: "Biological effects of low-level radiation", IAEA, Vienna, pp. 645-646

Strazhevskaya, N.B., Struchkov, V.A. (1977) Radiobiologiya, 17, 163-177

Szamel, M., Somogyi, J., Resch, K. (1982) Biokémia 6, 23-27

Szumiel, I. (1981) Adv. Radiat. Biol. 9, 281-321

Turner, J.E., Magee, J.L., Hamm, R.N., Chatterjee, A., Wright, H.A., Ritchie, R.N. (1980) Proc. 7th Symp. Microdosimetry, eds. Booz, J., Ebert, H.G., Hartfiel, H.D., Harwood, Ac. Publ. pp. 507-520

Wallach, D.F.H. (1974) Biomembranes 5, 213-249

Wolters, H., Konings, A.W.T. (1982) Radiat. Res. 92, 474-482

Acta Biochim. Biophys. Hung. 21/1-2/, pp.99-113/1986/

POSSIBILITIES OF BIOLOGICAL ENERGY PRODUCTION

L. Keszthelyi, Cs. Bagyinka, K. Kovács, I. Laczkó

Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

(Received September 12, 1984)

SUMMARY

From the numerous possibilities of biological solar energy transduction the production of hydrogen was selected in our laboratory. There are two forms of H_2 production: (i) from biomass with enzymes (formate-hydrogen lyase and hydrogenase enzymes) and (ii) the exploitation of some living systems or their analogs to evolve H_2 from water upon solar irradiation. To approach the first problem, a bacterial strain and a hydrogenase (H_2ase) enzyme of good parameters were isolated. The system composed produced H_2 from biomass with an energetic efficiency of 10-12 per cent. Dealing with the second task, by changing some environmental factors we succeded to increase by a factor of 15 the quantity of H_2 produced by Anabeana cylindrica from water in light.

It is clear from these studies that further research is needed to understand better the mechanism and regulation of biological H_2 production. The aim of this research is to utilize it as an economically feasible and environmentally harmless energy source.

INTRODUCTION

In recent years world-wide interest has arisen towards biologically based solar energy transduction. The reasons are well known: the prices of fossil energy sources (oil, gas, coal) are increasing and the known resources are sufficient only for a short period (ca 100 years), while the demand continuously increases because our life standard is determined mostly by the available energy.

The final supplier for the so-called renewable energy sources is solar energy. The Sun radiates 3×10^{24} J energy to the Earth surface every year. Let us call this one solar unit (S.U.). The result of the photosynthetic activity of the living systems is 10^{-3} S.U. energy stored in organic materials.

Mankind uses 10^{-4} presently mainly from fossil energy sources (food is about 5×10^{-6} S.U.). The estimated quantity of the economically feasible fossil energy is 10^{-2} S.U.
It may be seen that even with the very low efficiency of the global photosynthetic production 10 times more solar energy is transduced yearly than the present need. The reasons for the great interest is evident from the above numbers.

The use of biological solar energy transduction may be classified into two major categories:

1/ product oriented: the utilization of biomass from photosynthesis to produce energy-carrier substances:

2/ process oriented: the exploitation of the processes existing in living systems or their artificial analogs to transduce solar energy into electrical or chemical energy while not destroying the systems themselves.

One of the promising directions of energy carrier production is H_2 production. There are many advantages of an energy economy based on H_2 (Gregory, 1973). The most important is that the combustion of H_2 produces only non--polluting water.

The research reported in this paper deals with H_2 production from biomass (item 1) and by H_2 generating algae (item 2) where the source of H_2 is water.

The hydrogenase enzyme

The basic process in hydrogen production is the formation of H_2 molecule from 2 electrons and 2 protons

$$2e^- + 2H^+ \rightleftharpoons H_2.$$

This reaction is catalyzed by hydrogenase (H_2ase) enzymes in living organisms. These enzymes work also in the opposite direction (catalyze the decomposition of H_2 molecules to electrons and protons).

The presence of such enzymes in microorganisms has been known for more than 50 years (Maynew, O'Connor, 1982) although an intensive investigation of their properties, functions, and potential practical use started only about 10 years ago,

as a consequence of the first oil crisis.

H_2ase is an interesting protein from the point of view of general enzyme catalysis as well, since it catalyzes the reaction between the simplest possible substrates in biology, i.e., electrons and protons.

We chose photosynthetic bacteria as the source of hydrogenase since they can be cultivated relatively inexpensively using light as an energy source. This is a real advantage from the point of view of large scale production.

H_2ase in photosynthetic bacteria is generally believed to be coupled to N_2 fixation (Kondratieva, Gogotov, 1981) N_2 fixation is an extremely energy consuming process catalyzed by nitrogenase enzymes. This enzyme complex is involved in at least two functions: it produces H_2, and than consumes this H_2 for reducing N_2 to NH_3. H_2 is usually formed in excess in N_2 fixation and the assumed primary role of H_2ase in these microorganisms is to recycle H_2 into the N_2 fixation reaction.

Most of the photosynthetic bacteria possess H_2ase activity. For our studies, the purple sulfur bacterium Thiocapsa roseopersicina was chosen because of the outstanding stability of its H_2ase (Fig. 1, Bagyinka et al, 1981).

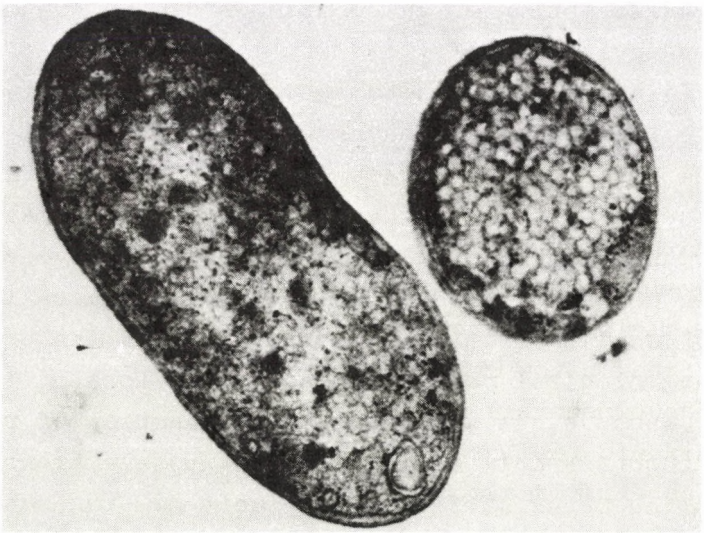


FIG. 1 The electronmicroscopic thin-section picture of Thiocapsa roseopersicina cells.

The location of the hydrogenase enzyme

On the basis of activity measurements on different cellular fractions (Table 1) H_2ase is believed to be located in the photosynthetic membrane of T. roseopersicina. Previously it was not known whether the active center of the enzyme faced the inner or outer side of the cell membrane. Since the answer to this question may reveal the physiological connection

TABLE 1 Distribution of H_2ase activity in T. roseopersicina

Sample	H_2ase activity (μmol $H_2 . h^{-1} . mg^{-1}$ protein)
Intact cell	3.0 ± 0.2
Protoplast	3.1 ± 0.2
Cytoplasmic fraction	0.4 ± 0.2
Membrane fraction	3.0 ± 0.2
Pure enzyme	51.3 ± 0.3

between the photosynthetic apparatus and the nitrogenase and H_2ase activities in these bacteria, a method has been worked out to determine the orientation of the H_2ase active center. A detailed description of these studies can be found elsewhere (Bagyinka et al., 1982).

Surprisingly, the results showed that the active center of H_2ase in T. roseopersicina as well as in several other photosynthetic bacteria (Kovács et al., 1983) is oriented toward the outer surface of the membrane. What does the mean with respect to the physiological function of the enzyme?

On the one hand, such an orientation of H_2ase is apparently puzzling from the point of view of its presently assumed physiological role. Nitrogenase is located in the cytoplasm. Thus, H_2ase can perform its function of recycling H_2 for nitrogenase only if it splits H_2 to electrons and protons at the outer surface of the cell membrane and the charged reaction products are then pumped back across the membrane (Fig. 2).

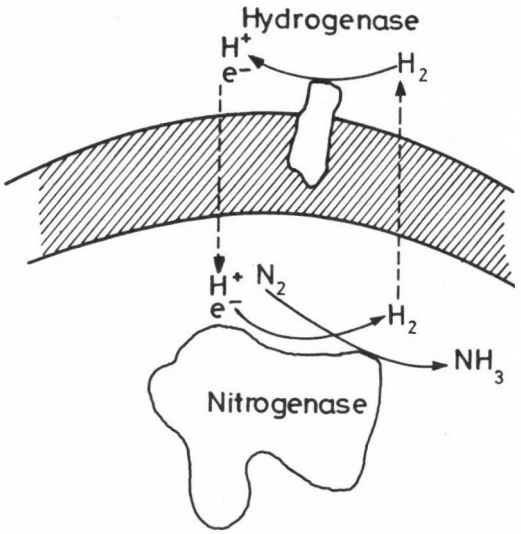


FIG. 2 The physiological relationship between H_2ase and nitrogenase in photosynthetic bacteria. The nitrogenase complex produces H_2 to reduce N_2 to NH_3. The excess H_2 is recycled by H_2ase.

On the other hand, it is known (Dér et al, 1983) that the direction of photosynthetic electron flow points toward the inner side of the membrane. Our measurements on the light induced primary charge movement in photosynthetic bacteria has corroborated this picture (Dér et al, unpublished). As H_2ase produces electrons at the outer surface of the same photosynthetic membrane it can be assumed that it couples to the photosynthetic electron transport chain by supplying electrons for it. Our understanding of the location of H_2ase has thus led to the assumption of hitherto unexplored possible functions of H_2ase in membrane bioenergetic processes.

Preliminary results (Bagyinka, Kovács, unpublished) substantiate this hypothetis. In order to get final evidence, recently monoclonal antibodies were produced (Tigyi, Kovács, unpublished) which selectively block the H_2ase <u>in vivo</u> and thus select those physiological reaction paths in which H_2ase takes part. (A specific inhibitor of this enzyme has not been available so far).

The work to reveal the role of H_2ase in the processes of photosynthetic pathways and in the highly energy consuming process of N_2-fixation is just beginning. It is believed that progress on this very important basic question will have significant impact on the strategy of developing new routes

for alternative energy production (either in N_2-fixation or H_2 evolution).

Fig. 3 sums up the processes leading to H_2 production and the key role of the H_2ase enzyme. Electrons and protons produced by other enzymes from biomass (process 1) or inside the cell by photosynthetic pathways or enzymatic decomposition of sugar (process 2) are synthetized to H_2 molecules by H_2ase and N_2ase enzymes.

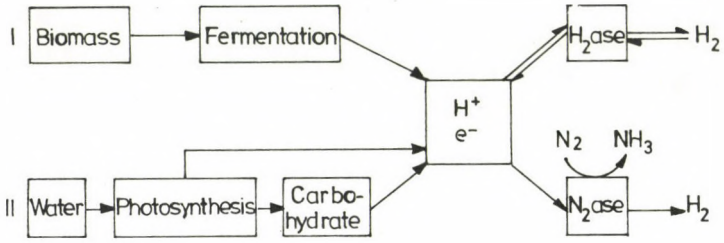


FIG. 3 H_2 evolution by reversible H_2ase from biomass via fermentation (I) and by nitrogenase from carbohydrate synthetized from water in photosynthesis (II). Hydrogenase operates exclusively as uptake H_2ase in nitrogenase catalysed H_2 production or N_2 fixation.

H_2 generation from biomass

Biomass may be defined as organic material of biological origin produced by solar energy through photosynthesis. From an energetic point of view it is a concentrated and storable form of low energy density solar radiation. Usually that part of organic material is considered as biomass which is not suitable for food. Biomass is used as raw material in the production of various energy carriers (ethanol, methane, H_2 etc.)

The results on H_2ase enzyme prompted us to work on H_2 generation from biomass. As raw material for biomass, first photosynthetic bacteria were tried because they grow in large quantities at low cost.

The principal requirement to fermentate biomass to H_2 is - according to Fig. 3 - the presence of the formate - hydrogen lyase enzyme and H_2ase in the system, in addition to the usual glycolytic enzymes. We have seen that T. roseopersicina contains an exceptionally stable H_2ase, it also has glycolytic

enzymes, but it does not synthesize formate-hydrogen lyase. Therefore, to study fermentative H_2 production an Enterobacter sp. straing isolated and purified in our laboratory was introduced into the system. The Enterobacter sp. shows high formate-hydrogen lyase activity. It has also been found that cell-free extracts of T. roseopersicina can serve as a good medium for growth of Enterobacter sp. The steps in looking for an efficient system were the following: intact T. roseopersicina cells repressed in nitrogenase synthesis (Kondratieva et al., 1982) are unable to generate H_2 even in the presence of Enterobacter sp. cells. Traces of H_2 are observed when the cells are supplied with exogenous glucose. When both methylviologen as an electron carrier and glucose are added to the cells, the H_2 generation increased by a factor of about 20 (Table 2). This indicates that the reducing power formed in glucose fermentation needs electron mediators to be carried over to the H_2ase enzyme.

TABLE 2 H_2 production by T. roseopersicina and its fractions after incubation at 37°C for 72 hours

[a]Glucose concentration: 40 mg/ml methylviologen (MV) concentration: 10^{-3}M

[b]Cells were disrupted by sonication at +4°C, under N_2

[c]Supernatants were prepared from the sonicated cells by centrifugation at 14°C for 2 hours.

Sample	H_2 production (μmol $H_2 . ml^{-1}$)
Intact cell	0.0
Intact cell + glucose[a]	0.4
Intact cell + glucose + MV	11.6
Sonicated cell[b]	0.0
10^4 x g supernatant[c]	17.8
10^5 x g supernatant[c]	16.8

It was observed that starving cells use up the H_2 they have previously produced under optimum growth conditions. (This is in accordance with results of Seifert and Pfennig (1978)).

A considerable increase in H_2 generation is obtained when a cell-free extract of T. roseopersicina is used in the system instead of intact cells. Thus our basic fermentative H_2 producing system consists of a cell-free extract of T. roseopersicina inoculated with Enterobacter sp.

The basic system is capable of H_2 generation from the organic materials present in the T. roseopersicina extract. Addition of DNA or cellulose to the basic system does not stimulate H_2 production (Table 3). When partially hydrolized starch or protein is added, the H_2 production increases.

TABLE 3 H_2 production by the basic system from various substrates after 72 hours of incubation at 37°C.

[a]The substrates were added in 40 mg/ml initial concentration

Sample[a]	H_2 production (%)
Basic system (BS)	100
BS + DNA	105
BS + Cellulose	97
BS + Starch	121
BS + Bovine serum albumin	178
BS + Glucose	494

Addition of glucose or other monosaccharides or disaccharides increases H_2 formation dramatically. From 1 liter of cell-free extract (ca. 15 mg protein/ml) about 2.5 liter/g of H_2 gas can be produced at a starting glucose concentration of 40 mg/ml. The yield of glucose conversion to H_2 is stable even at high initial concentrations of the substrate (Fig. 4).

Although it is not optimized yet, this H_2 generating system routinely yields 1.2-1.3 mol H_2/mol glucose which

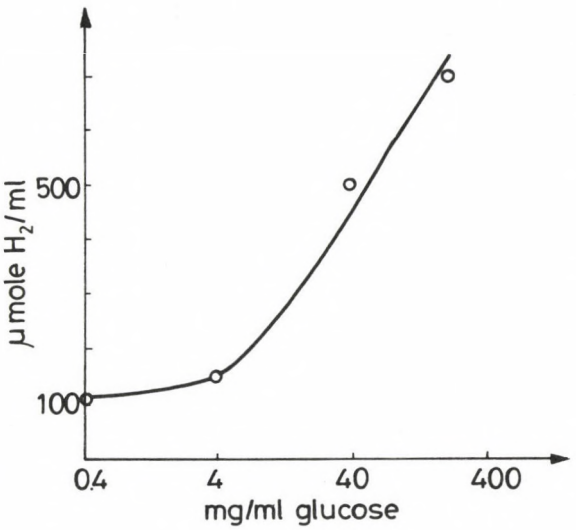


FIG. 4 H_2 generation in the basic fermentative H_2 producing system (see text) as a function of glucose concentration. In the presence of small amount of glucose H_2 is produced from the biomass of cell-free extract. The increase in H_2 partial pressure and in osmotic pressure due to high glucose concentration apparently does not affect H_2 production.

corresponds to 11 per cent energy conversion. The theoretical upper limit is 4 mol H_2/mol glucose, i.e., 33 per cent of the chemical energy stored in glucose can be converted into H_2. On the basis of energy balance fermentation of sugars in biomass to H_2 is not competitive with biogas reactors (in which the energy conversion yield is routinely about 60 per cent, the theoretical upper limit is 85 per cent, i.e. 3 mol CH_4/mol glucose). Sugar fermentation, however, produces H_2 at similar energy conversion yield as ethanol fermentation does. In the latter case a diluted water-ethanol mixture is formed from which water should be removed by energy consuming distillation. Nevertheless, large scale ethanol production is considered to be economically feasible only under certain geographical and economic conditions.

The H_2 generating fermentative system outlined above may be used for energy production at least as well as fermentation of the same biomass to ethanol. The outstanding features of this system which make in suitable for such purposes can be summarized as follows:

1. It is a simple, inexpensive form of H_2 production.
2. It can generate H_2 from various organic materials (especially mono- and disaccharides, proteins and certain organic acids are good substrates). In addition, the initial substrate

concentration and the accumulated product to not affect the H_2 producing ability over a wide concentration range.

3. It is stable in time, after several days of storage the basic system is able to work at the same rate (upon addition of substrates) as the freshly prepared one (Fig. 5).

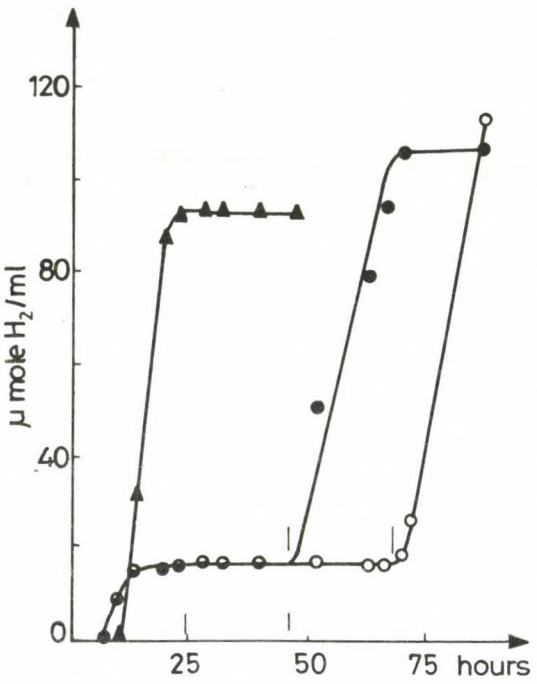


FIG. 5 The time course of H_2 production in the basic fermentative system. Arrows indicate the addition of 40 mg glucose, vertical bars show the replacement of the atmosphere for Ar. The initial 8-10 hours lag period is needed for the Enterobacter sp. growth. In samples (II and III) where glucose was added after long preincubation and initial H_2 production from cell-free extract is observed. H_2 production can be started even after 3 days (Sample III) of pre-incubation with the same rate and yield as in sample I.

H_2 generation by algae

As it has already been mentioned, the nitrogenase enzyme produces H_2 as a secondary product. The final source of H_2 in algal systems is water splitting. We chose the filaments of a heterocystous blue-green alga, Anabaena cylindrica for our studies.

The blue-green algae are the only organisms which combine green plant photosynthesis and nitrogen fixation. They have evolved the mechanism which allows the coexistence of these two, incompatible reactions: in the course of photosynthesis O_2 is produced, which inhibits the activity of the nitrogenase.

The filaments contain two kinds of cell (Fig. 6)

1./ Vegetative cells that perform photosynthesis like the

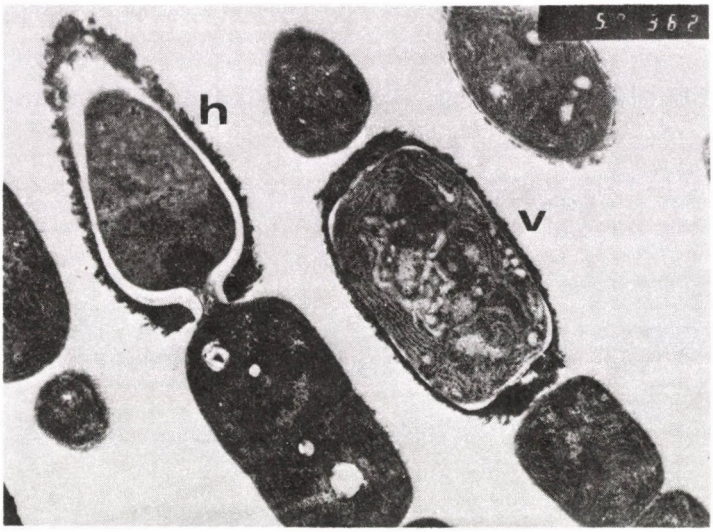


FIG. 6 The electronmicroscopic thin-section of Anabaena cylindrica: h and v denote heterocyst and vegetative cell, respectively.

higher-plants (they contain the two photosystems PSII, PSI). The water splitting reaction, O_2 evolution and CO_2 fixation take place in these cells.

2./ Heterocysts: under nitrogen-fixing conditions about every 15th vegetative cell differentiates into a heterocyst. They have a bacterial-type of photosynthesis (PSI). The available evidence suggests that the heterocyst is the site of nitrogenase activity. The necessary reductant is provided by transport of reduced carbon compounds from vegetative cells (which in return, receive fixed nitrogen for protein synthesis from heterocysts).

Under an inert gas these algae would function as a biophotolytic system, with H_2 produced by the nitrogenase enzymes of the heterocysts and O_2 by the vegetative cells. However, the amount of H_2 produced in this way by aerobically grown A. cylindrica is small.

Our investigation aimed at increasing the H_2 generation by altering the environmental conditions. It turned out that the low rate of H_2 production of these algae could be enhanced by nitrogen starvation. The algal culture was kept under argon atmosphere for several days. The rate of H_2 generation,

nitrogenase activity (measured as formation of ethylene from acetylene) and heterocyst frequency were measured on each day of starvation (Fig. 7). The maximum heterocyst frequency (12 per cent and nitrogenase activity developed on the second day of starvation: twice as much ethylene was produced, relative to t=0 value. While the nitrogenase activity doubled only,

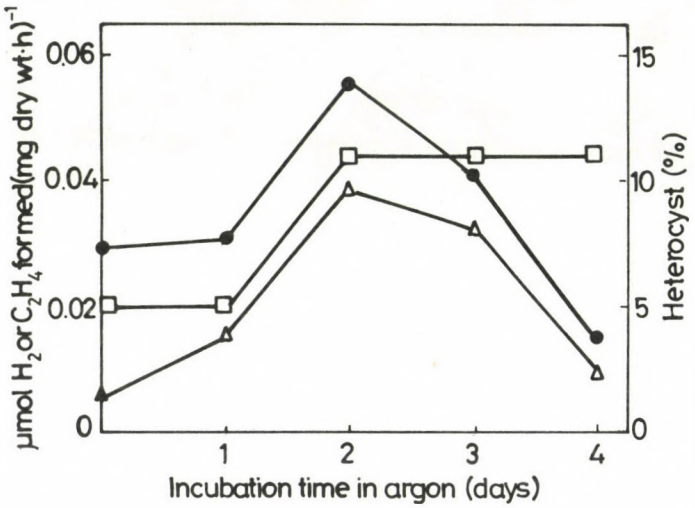


FIG. 7 Effect of nitrogen starvation on heterocyst frequency (—□—), nitrogenase activity (—●—) and hydrogen generation (—△—) by Anabaena cylindrica. Cells (0.1 mg dry wt.ml^{-1}) were suspended in 20 mM Tris-HCl buffer, pH 7.4. Light intensity: 100 W.m^{-2}.

the rate of H_2 formation increased 5-fold. To interpret the results one has to know that an H_2ase enzyme can also be found in the heterocysts (Fig. 3) which, working in the reverse direction, recycles the H_2 molecules for further nitrogen fixation. Hence the H_2 formation depends on the balance of the activity of N_2ase and H_2ase enzymes. The explanation of the increased H_2-generation is that during nitrogen starvation the activity of the H_2ase decreases (Laczkó, 1980).

In other experiments the H_2 generation of photobleached cells of A. cylindrica possessing higher photosynthetic capabilities was studied. We found that cells grown at high light

intensity (100 W/cm^2) became orange-yellow due to decomposition of chlorophyll and phycocyanin and heavy accumulation of carotenoids (Laczkó, Barabás, 1981). In these photobleached cells an elevated rate of H_2 production accompanied by a higher rate of water splitting was observed. Under argon atmosphere photobleached cells produced H_2 at 5 times the rate of normal cells. Since the increase of H_2ase activity was smaller, we have to suppose the presence of another enzyme to catalyze H_2 production in photobleached cells. The considerable H_2 generation even in a nitrogen atmosphere proved that during photobleaching a reversible hydrogenase activity was induced. 3-(3',4'-dichlorophenyl) (-1,1-dimethyl-urea (DCMU) inhibits this H_2 production indicating that water is the source of H_2 and electrons.

Further studies were performed to explain the activation of the reversible hydrogenase enzyme in photobleached cells. The rate of photosynthetic O_2 production increased in photobleached cells, however, such an increase in CO_2 fixation could not been observed (Fig. 8) which means that many

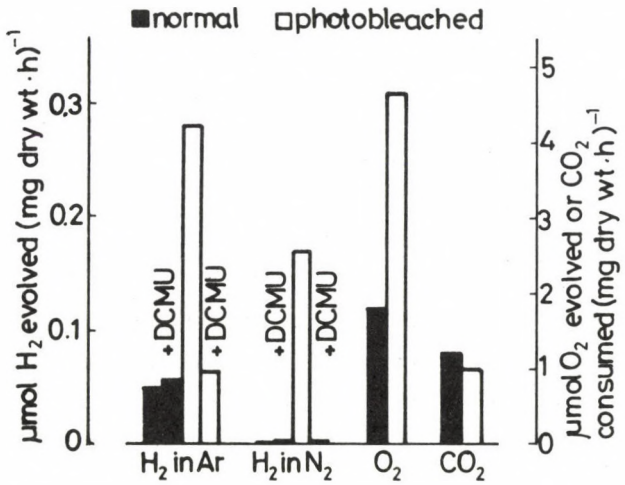


FIG. 8 H_2 and O_2 production and CO_2 consumption by Anabaena cylindrica. Cells (0.1 mg dry wt. ml^{-1}) were suspended in 15 mM Tris-HCl + 5 mM $NaHCO_3$, pH 7.4, DCMU: 2.10^{-5} M. Light intensity: 100 W.m^{-2}.

electrons entering the photosynthetic electron-transport chain are not used for CO_2 fixation. The results show that these electrons and the associated H^+ ions are converted to H_2 by the H_2ase enzymes induced during photobleaching. Hence the energy producing H_2 generation sustained for long periods

can be assumed to be a protective mechanism against over-reduction of the photosynthetic electron transport chain.

Summerizing, the increased H_2 generation in photobleached Anabaena cells is the result of an increased nitrogenase and an induced reversible hydrogenase activity.

Although 10 per cent conversion of solar energy into chemical energy can be theoretically achieved with photosynthetic process, the actual conversion in practice is not higher than 2 per cent in our system. The experimental results show (Table 4) that the efficiency can be substantially increased in the photobleached cells of Anabaena cylindrica, as compared to that of normal cells.

TABLE 4 Efficiency of light $\longrightarrow H_2$ energy in Anabaena cylindrica grown under different conditions

Algae	efficiency (%)
Normal	0.1
N_2 starved	0.3
Photobleached	1.5

Conclusions

Although the results are promising, it is obvious that at this stage further intensive basic research is urgently needed to reveal more precisely the underlying molecular mechanisms so as to understand the regulation of H_2 production in biological systems and enhance its efficiency. With this knowledge the biological energy producing processes can be utilized as an economically feasible and environmently safe alternative energy source on a large scale.

REFERENCES

Bagyinka, Cs., Dancsházy, Zs., Kovács, K.L., Ormos, P., Keszthelyi, L. (1981) Acta Biol. Acad. Sci. Hung. 32, 311-325

Bagyinka, Cs., Kovács, K.L., Rák, E. (1982) Biochem. J. 202, 255-258

Dér, A., Bagyinka, Cs., Kovács, K.L. (1983) Proc. Int. Symp. on Solar Energy Bioconversion. Puschino (in press)

Gregory, D.P. (1973) Sci. American 228, 13-21

Kondratieva, E.M., Gogotov, I.N. (1981) Nauka p. 216

Kondratieva, E.M., Ivanovsky, R.N., Krasilnikova, E.N. (1982) Soviet Sci. Rev. D2, 325-364

Kovács, K.L., Bagyinka, Cs., Serebriakova, L.T. (1983) Curr. Microbiol. 9, 215-219

Laczkó, I. (1980) Z. Pflanzenphysiol. 100, 241-245

Laczkó, I., Barabás, K. (1981) Planta 153, 312-316

Maynew, S.G., O'Connor, M.E. (1982) TIBS 7, 18-21

Seifert, E., Pfennig, N. (1978) Biochimice 60, 261-265

Acta Biochim. Biophys. Hung. 21/1-2/, pp.115-128/1986/

STRUCTURE OF THICK FILAMENTS FROM INSECT FLIGHT MUSCLE

K. Trombitás, A. Tigyi-Sebes

Central Laboratory, Medical University, Pécs, Hungary

(Received July 9, 1985)

SUMMARY

The supramolecular organization of thick (myosin) filaments isolated from insect flight muscle was studied using negative staining and shadowing techniques. The electron microscopical findings favour the two-stranded arrangement of double cross-bridges rather than a four- or six-stranded structures of single cross-bridges. The thick filament backbone consists of 12-sub-filaments of myosin rods with a diameter of about 4 nm in agreement with the X-ray data. Furthermore, about 4 nm stripping was observed on the filament shaft, resembling to the structure of the light meromyosin paracrystals with a periodicity of 4.8 nm. The existence of a hinge region at the origin of the projections and between the tail and myosin heads are confirmed. According to the morphological observations the stalks of the projections can be characterized with flexible properties, as well. At high level of the supra-molecular organization, the myosin filaments, the connecting filaments and the Z-filaments are systematically interconnected after a remarkable myosin filament branching (trifurcation) at the Z line level, which supports the view that a continuous longitudinal filament network constitutes the structure of the myofibrils.

INTRODUCTION

Despite of the great efforts in the latest time the structure of the thick filaments of insect flight muscle remained questionable (Heuser, 1983). Currently, for the arrangement of the myosin projections three different helical models were proposed. The first model was based on the static array of cross-bridges in rigor, which was revealed by electron microscopy (Reedy, 1967; 1968; Reedy, Garret, 1977). Using Reedy's excellent micrographs it was suggested that the array

of the myosin projections along the thick filaments could be described by a two-stranded helix with 116 nm true repeats and 14.5 nm subunit axial translation. The X-ray diffraction pattern could also be interpreted in terms of the two-stranded helix of cross-bridges (Miller, Tregear, 1972). Recently this two-stranded helical model has been reconsidered by the investigation of the freeze-etching pattern of the insect flight muscle (Heuser, 1983). The number of myosin molecules per 14.5 nm repeat (per crown) along the thick filaments proposed for insect flight muscle is either six (Chaplain, Tregear, 1966; Bullard,Reedy, 1973; Reedy et al., 1973; Tregear, Squire, 1973), or four (Wray, 1979; Reedy et al., 1981). Theoretical considerations (the general model for the myosin filament structure) were based on the evidence that the number of projections per crown should be six (Squire, 1972). In this way a six-stranded, right handed helix model was constructed with a 231 nm pitch and 115.5 nm repeat (Squire, 1977, 1981).

Recent papers indicating four myosin molecules per crown support Wray's hypothesis (1979) which considers a four-stranded arrangement in the thick filaments of insect flight muscle (Freundlich, Squire, 1983; Taylor et al., 1984; Haselgrove, Reedy, 1984). On the basis of the X-ray diffraction findings none of the discussed models could be excluded (Squire, 1981).

Therefore, it seems desirable to perform high resolution electron microscopic studies on isolated myosin filaments. Isolated filaments were prepared first by Huxley (1963) from glycerinated rabbit psoas muscle by intensive homogenization in relaxing medium. Since this method was not successful for the insect flight muscle because of the stabilizing effect of the continuous transverse structures such as the M and Z line components, Garamvölgyi and Trombitás (1966) proposed the specific removal of the M and Z line material before the filament dispersion. This isolation method with certain modification was then generally used (Morimoto, Harrington, 1973; Reedy et al., 1981), and different techniques were applied for investigation of the isolated thick filaments. Reedy and Garret (1977) showed a 14.5 nm transverse periodicity on the

negatively stained Lehocerus thick filaments. This periodicity represented the myosin heads along the filament surface. Using the freeze-drying and shadowing method, individual myosin molecules were presented on rabbit psoas thick filaments by Trinick and Elliot (1979). The two-headed myosin molecules surrounded the filament shaft, which appeared to be relatively smooth. When the negative staining technique was combined with the shadowing procedure, both the individual projections and the surface structure of the filament shaft could be demonstrated (Trombitás, Tigyi- Sebes, 1980). Knight and Trinick (1984) visualized rabbit myosin filaments by a negative staining method and reported on the size and shape of the head of the projections as well as the flexible properties of them. Nevertheless, the helical arrangement of the projections remained unanswered. The greatest progress was made by Stewart et al. (1981) and by Kensler and Levin (1982) who were able to reconstruct the four-stranded helices of Limulus muscle by computer analysis of the electron micrographs of negatively stained and shadowed thick filaments. Vibert and Craig (1983) using the same method, pointed out that the cross-bridge array of the myosin filaments from scallop striated muscle has 7-fold rotational symmetry. Nevertheless, nobody could tell so far anything about the individual origin of projections. In this paper we present new results on the properties of the myosin helices, the peculiarity of the projection origins, the flexibility of the myosin projection, and the structure of the filament backbone.

MATERIALS AND METHODS

Thick filaments from the honey-bee (apis mellifera) flight muscle were isolated according to the method of Morimoto and Harrington (1973) with slight modification. The fresh dorso-ventral flight muscles were stretched and soaked in low ionic strength medium consisting of 5 mM TRIS-HCl and 10 mM dithiothreitol, pH 7.8 at 2^{o}C. After 24 hours incubation the muscles were homogenized and centrifuged at 1.000 g for 10 minutes. The sediment was then resuspended again in the same

low ionic strength solution. This procedure was repeated for 7 or 14 consecutive days. The first period was sufficient to extract completely the M line material and partially the Z line material. The prolonged extraction removed the Z lines completely. Usually a gap or split occurred in the centre of the A band at the level of the extracted M lines (Goll et al., 1977). Finally, the myofibrillar material was centrifuged and resuspended in relaxing medium containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM ATP, 20 mM potassium phosphate at pH 7.0. This suspension was homogenized three times for 20 seconds in a Waring blendor. The intact fibrils were centrifuged at 3.000xg for 15 minutes. The turbid supernatant contained the isolated filaments. The thick filaments were separated from the thin filaments by zone sedimentation in a density gradient of glycerol (Morimoto, Harrington, 1973). The supporting solution was the relaxing medium and a linear gradient from glycerol. Its concentration varied in the zone between 10 per cent from the top to 30 per cent from the bottom in the centrifuge tube. A 4 ml aliquot of the filament suspension was layered in each tube containing 32 ml supporting solution. The samples were centrifuged in a Spinco SW-27 swinging bucket rotor at 22.000 rpm for 2 hours. Six equal volume fractions were collected from each tube, and studied by SDS polyacrylamide gel electrophoresis (Weber, Osborn, 1969). Thick filaments were found in the first two fractions, thin filaments in the fourth and fifth fractions (Trombitás, Tigyi-Sebes, 1979). The separated thick filaments were stored in 30 per cent glycerol at $-20^{\circ}C$ until use.

A drop of filament suspension was applied to a grid coated with carbon film and it was negatively stained with 1 per cent uranyl acetate. The negatively stained and air dried filaments were both rotary and unidirectionally shadowed with platinum-palladium vapour (ratio 3:1) at an angle of 15° (Trombitás, Tigyi-Sebes, 1980; see also Levin and Kensler, 1982; Vibert, Craig, 1983). The uranyl acetate fixation preserves the fine structural details of the native filaments, decreases the effect of drying procedure and the

high resolution shadowing makes the fine details more visible.

The grids were inserted in the electron microscope with the specimen side towards the electron source. The micrographs were either enlarged so that the emulsion surface of each negative was kept uppermost in the photo enlarger (Reedy, 1968), or reversed by contact printing together with the emulsion sides and the final enlargments were made with the emulsion side of the reversal away from the light source producing the correct handedness of the filaments (Vibert, Craig, 1983).

RESULTS

The extraction of the M and Z lines based on the modification of the extracting method of Morimoto and Harrington (1973) proved to be successful. Figs. 1a and b show the progressive removal of the M and Z line material during the first and the prolonged extracting periods. The M line material and the central zone of the myofibrils were extracted completely before the complete Z line extraction occurred. Generally, the myofibrils were fragmented into about one-sarcomere-sized parts, in which the two half-sarcomeres were connected with the rest of the Z line material. It seems that the extraction was effective even at the central part of the myofibrils, i.e. at the "bare zone" of the thick filaments. After the extraction periods, the filaments could be dispersed easily in relaxing medium and relatively pure thick and thin filament fractions could be separated with the gradient centrifugation (Figs. 2a, b). The SDS polyacrylamide gels demonstrate that the extraction procedure did not cause essential digestion effect on the filament systems, furthermore, the thick filaments contained a relatively large amount of paramyosin (Fig. 3).

Full length thick filaments from the shorter extraction procedure showed regular periodicity after rotary shadowing (Fig. 4). It seemed that the structure of the filaments was intact, except in the central zones where the filaments were tapered in a very regular manner. It is thought that the thinner strips represented the helical gyres, the wider strips contained perhaps two associated gyres. The triangular profils

(white arrows) indicated the possible helical arrangement of the projections (Fig. 5), which could rarely be observed directly (white arrowhead). One of the filaments had an interesting structure: the one and a half sarcomere long filament bifurcated at the one third of its length, where the joining filament ends were very regularly tapered. The same phenomenon could be frequently observed in negatively stained samples (Fig. 6). It was supposed that the filaments were continuous because of the existence of C filaments and that they bifurcated at the level of the Z lines (Trombitás, Tigyi-Sebes, 1979).

In samples shadowed unidirectionally, only the filaments lying parallel or perpendicular to the direction of the shadowing were taken into account. The peaks of projections produced by perpendicular shadowing of the filaments showed a periodicity of about 38 nm with a limited perturbation (Figs. 7a, b). A thin filament shadowed similarly supplied as with a fairly good reference periodicity value of 38.5 nm (Fig. 7c). To decide the right projection arrangement, the optical shadowing of the proposed models was compared with the micrographs (Figs. 7d, e, f). The models of the two--stranded helices gave an optical shadow peak repeat of about 40.0 nm, which may be compared to shadow pattern, observed on electron micrographs, while the models, both the four-, and six-stranded helices, produced shadown with periodicities of 14.5 nm. Along the parallel shadowed filaments (Figs. 8a, b, c, d) the projection arrangement could also be characterized with periodicity of about 38 nm. The absolute handedness of the helices could not be determined unambiguously because of the association tendency of the projections, but it seemed quite probable that the helices were left handed (Figs. 8a, c; black arrows). On some occasions individual projection origins could be seen on the filament shafts, which were arranged on a left handed helical track (Fig. 8c, black arrow). The elongated large heads of the projection lie very regularly beside each other. Moreover, pairs of the projections in the fringe, frequently appeared

to originate from a common point (Fig. 8c doubled arrows). The individual projections, which seemed to be too large for one myosin molecule, were arranged along regularly repeating helical gyres on the filament shaft (Fig. 9). The arrows point to the possible alternate helical gyres, which are spaced about 2x38 nm apart. The full length filament was trifurcated in its center making an impression that one filament could continue into three different filaments. After bifurcation or trifurcation the filament pairs always ran far from each other. This phenomenon indicates perhaps the Coulombic interaction of the projections.

After prolonged extractions, the M and Z material with the bare zones of filaments were completely extracted. Although, a certain number of the projections were lost during the preparation, the rest of the projections seemed to be intact. Therefore, it was possible to investigate the individual projections, both the heads and the stalks, as well as the backbone structure of the filaments. All the projection heads were adhered to the filament shaft (Fig. 10a, b). The filament backbone was composed of five longitudinal subfilaments, which appeared to have very fine transverse periodicity. The complete projections (black arrows) were too large for single myosin molecules (double arrowheads, fig. 10b). Furthermore, pairs of projections appeared to originate from common points (white arrowhead). The stalks of the projection, which correspond to the subfragment 2 of the myosin molecules were separated from the filaments but turned around the shaft. The individual heads could attache to the shaft in different angles (Figs. 11a, b, c, d). Occasionally, projections composed of more then two myosin heads with paired stalks could also be seen (Figs. 12a, b arrows). The filament shaft showed a fine transverse periodicity of about 4 nm (Figs. 13a, b see also Fig. 11c).

DISCUSSION

It was discussed already that in rigor the cross-bridge lattice reflects the symmetry of the thin rather than the thick filaments (Huxley, Brown, 1967; Squire, 1972),therefore it seemed reasonable to investigate isolated filament system. To avoid the disturbing effect of the actin-myosin interactio after longer storage, in which case the cross-linking of the heads and backbone might be expected to exhibit directly the cross-bridge symmetry, the thick filaments were separated from the thin ones (Trombitás, Tigyi-Sebes, 1979, 1980).

It seems to be worth to discuss the findings on thick filaments isolated from insect flight muscle under the following headlines: a, the helical arrangement of the projections; b, filament branching; c, the backbone structure of the thick filaments, d, the flexibility of the projections; e, paramyosin content of the thick filaments.

a, The helical arrangement of the projections

In the seventies the arrangement of the projection origins on the myosin filaments was very strongly disputed. Reedy's group preferred the two-stranded double cross-bridge model, while Squire's group argued for the six-stranded single cross-bridges (e.g. Reedy, Garret, 1977; Squire, 1977). Reedy's view was based on his exceptionally beautiful micrographs (the flared X cross-bridge configuration), while Squire's argument was supported by the myosin content of thick filaments, i.e. six myosin molecules per crown. To a certain extent, both versions were accepted. When Wray (1979) proposed the four--stranded model for the insect flight muscle and Reedy et al. (1981) reinvestigated the myosin content of thick filaments, illustrating four myosin molecules per crown, the most evident thing was to accept the right handed, four-stranded single cross-bridge model (Freundlich, Squire, 1983; Taylor et al., 1984; Haselgrove, Reedy, 1984).

In the meantime the freeze fracture and deep-etching technique provided us with new findings. Heuser (1983) was

able to demonstrate Reedy's earlier model: the two-stranded left handed helical cross-bridge pattern in rigor. Heuser's recently published results strongly support our work previously published in a short form (Trombitás, Tigyi-Sebes, 1980).

Both the rotary and the unidirectionally shadowed filaments show a distinct periodicity of about 38 nm. The periodic stripes correspond to the helical gyres, although the helical order is quite disturbed, therefore it is difficult to recognise the handedness. Really there are some uncertainties about the orientation of the stripes, and the handedness can not be determined unambiguously, but it seems more likely that the helices are left handed. Considering the helical order of the projections, the 38 nm repeats formed by associated projection heads are too large for a four-stranded helical model, as it was shown by the optical shadowing (Figs. 7d, e, f). The parallel-shadowed filaments clearly show that the successive gyres are spaced about 38 nm apart (Figs. 8, 9). Therefore, the essential conclusion of the study is that the cross-bridge origins are arranged on the thick filament shaft along two-stranded rather than four-, or six-stranded helical tracks.

On the other hand, in some regions, where the projection origins can directly be seen on the filament shaft, there are two projections close to each other, which probably means that the projections protrude in symmetrical double-pairs i.e. four of them per crown. Furthermore, the individual projections heads are too large for ony myosin molecule. This contradiction could be solved by presuming that the heads (four subunits) of the doubled myosin molecules were associated.

b, The filament branching

The thick filament bifurcation previously observed in the Z lines have been used as a proof of the filament continuity across the Z line (Trombitás, Tigyi-Sebes, 1979). After observing the trifurcated forms of the thick filaments, a new

Z line structure was suggested. In this model, every thick filament is connected with three different thick filaments from the adjacent sarcomere, and these connections at the Z lines result in the characteristic oblique appearance of the Z filaments (Fig. 1a arrow; Trombitás, Tigyi-Sebes, 1980). The oblique appearance of the Z filaments comes from the fact that the arrangement of the thick filaments in the two adjacent sarcomeres are in different planes (Ashhurst, 1967). This idea could be supported by micrographs taken from very thin longitudinal sections. Nevertheless, our explanation for branching has been criticized. The basis of the critiques emerged from the impression that the filament branching would occur in the M line region. Really the thick filaments have an oval profile in the M region (Auber, 1967; Squire, 1977) and such oval profile can often be resolved into two more-or-less distinct circles lying side by side. Recently Heuser (1983) cleared up the M line phenomenon and concluded that the myosin filaments split into two distinct subfibers in the M region, but these subfibers build up the same filament. Our recent findings support also the thick filament branching at the Z line. If we supposed that the filament branching occurs at the M region, we would have difficulties in the explanation of the one and a half-sarcomere long filaments. Fig. 5 and 6 support the original conception, namely that the thick filaments are continuous across the Z line and every thick filament is connected at least with three different thick filaments from the neighbouring sarcomeres. This observation means that a continuous longitudinal filament network exists and takes part in the construction of the myofibrils.

c, The backbone structure of the thick filaments

It was shown earlier that the backbone of myosin filaments contain resoluble structures. Gilev reported (1964, 1966; Gilev et al., 1968) that the myosin filaments consisted of 12-18 subfilaments in the crab muscle. Davey and Graafhuis (1976) presented interesting results on the structure of isolated thick filaments taken from hen pectoral muscle.

The isolated filaments were treated with trypsin to remove the myosin heads, and the resulting micrographs gave evidence for the backbone structure of three intermediate filaments. Each intermediate filaments contained three or more subfilaments. Wray (1979) also obtained important findings on the structure of the myosin filament backbone. His findings consist of observations of X-ray reflections at radial positions about 4 nm from the meridian which reflexions form part of the myosin layer lines. The reflections remained intense in pattern from rigor muscle, which made them conclude that the subfilaments of myosin rods with a 4 nm diameter are paked closely with their neighbours. Applying this subfilament model to the myosin filaments of insect flight muscle, the 20 nm diameter of a single myosin filament observed by electron microscopy could accomodate 12 subfilaments within a single myosin filament. In this case five subfilaments could be observed on the surface of the filament. Our micrographs strongly support this hypothesis showing five subfilaments along the backbone of a myosin filament (Figs. 10a, b).

A very fine transverse periodicity of about 4 nm is superimposed onto the subfilaments, which probably reflects the molecular packing of myosin rods. This phenomenon shows similarity to the LMM paracristals with a 4.8 nm axial periodicity, which was first presented by Podlubnaja et al. (1969).

In conclusion, the presented results suggest that the thick filaments of insect flight muscle are composed of 12 subfilaments, and the doubled myosin molecules are arranged in a two-stranded helical configuration.

d, The flexibility of the projections

Huxley (1969) suggested that the myosin projections had hinge region at their origin as well as between the heads (subfragment 1) and the stalk (subfragment 2) making them function as cross-bridges. The flexible properties of the projections were demonstrated either by visualization of separated myosin molecules and myofilaments (Sleyter, Lowy, 1967; Elliot, Offer, 1978; Knight, Trinick, 1984), or by

investigation of the intact muscle in rigor or under relaxed condition (Reedy et al., 1965; Reedy, 1967, 1968). As Figs. 10, 11, 12 show the projections can fold around the shaft and attach to the filament backbone in different angles. This phenomenon proves the presence of a flexible joint at the head-tail junction and at the origin of the projections. Furthermore, the stalks (subfragment 2) seem to be sufficiently flexible to allow all the cross-bridges to reach the target areas on the thin filaments. Therefore, it is very probably that in rigor all the projections form cross-bridges, as it has been shown in thin sections (Reedy, 1968), or in freeze etching replicas (Heuser, 1983).

e, The paramyosin content of the thick filaments

The insect flight muscle contains a large amount of paramyosin (Bullard et al., 1973). Immunohistological investigations have proved that paramyosin was always located in the thick filaments (Levin et al., 1974; Bullard et al., 1977a, b; Trombitás et al., 1977; Bullard, 1983). These findings were confirmed by the SDS polyacrylamide gel electrophoresis performed on quite pure thick filament fraction (Fig. 3). The paramyosin content of the purified thick filaments was significant.

Acknowledgements

The authors are indebted to Dr. M. Kellermayer for reading the manuscript and for many helpful discussions.

REFERENCES

Ashhurst, D.E. (1967) J. Mol. Biol. 27, 385-389

Auber, J. (1967) Amer. Zool. 7, 451-456

Bullard, B. (1983) Trends Biochem. Sci. 8, 68-70

Bullard, B., Bell, J.L., Luke, B.M. (1977) In: Insect Flight Muscle (Tregear, R.T. ed.) pp. 41-52, North Holland, Amsterdam

Bullard, B., Hammond, K.S., Luke, B.M. (1977) J. Mol. Biol. 115, 417-440

Bullard, B., Luke, B.M., Winkelman, L. (1973) J. Mol. Biol. 75, 359-367

Bullard, B., Reedy, M.K. (1973) Cold Spring Harbor Symp. Quant. Biol. 37, 423-428

Chaplain, R.A., Tregear, R.T. (1966) J. Mol. Biol. 21, 275-280

Davey, C.L., Graafhuis, A.E. (1976) Experientia 32, 32-34

Elliot, A., Offer, G. (1978) J. Mol. Biol. 123, 505-519

Freundlich, A., Squire, J.M. (1983) J. Mol. Biol. 169, 439-444

Garamvölgyi, N., Trombitás, K. (1968) Symp. Biol. Hung. 8, 114-119

Gilev, V.P. (1964) Biochim. Biophys. Acta 79, 364-368

Gilev, V.P. (1966) Biochim. Biophys. Acta 112, 340-346

Gilev, V.P., Perov, N.A., Yu, E. (1968) Arh. Anat. Gist. Embriol. 54, 41-51

Goll, D.E., Stromer, M.A., Robson, R.M., Luke, B.M., Hammond, K.S. (1977) In: Insect Flight Muscle (Tregear, R.T. ed.) pp. 15-40

Haselgrove, J.C., Reedy, M.K. (1984) J. Muscle Res. Cell Motil. 5, 3-24

Heuser, J.E. (1983) J. Mol. Biol. 169, 123-154

Huxley, H.E. (1963) J. Mol. Biol. 7, 281-308

Huxley, H.E. (1969) Science 164, 1356-1366

Huxley, H.E., Brown, W. (1967) J. Mol. Biol. 30, 383-434

Kensler, R.W., Levine, R.J.C. (1982) J. Muscle Res. Cell. Motil. 3, 349-361

Knight, P., Trinick, J.A. (1984) J. Mol. Biol. 177, 461-482

Levine, J.D., Dewey, M.M., Elfvin, M., Walcot, B. (1974) Amer. J. Anat. 141, 453-458

Levine, R.J.C., Kensler, R.W. (1982) Biophys. J. 37, 50a

Miller, A., Tregear, R.T. (1972) J. Mol. Biol. 70, 85-104

Morimoto, K., Harrington, W.F. (1973) J. Mol. Biol. 77, 165-175

Podlubnaya, Z.A., Kalamkarova, M.B., Nankina, V.P. (1969) J. Mol. Biol. 46, 591-592

Reedy, M.K. (1967) Amer. Zool. 7, 465-481

Reedy, M.K. (1968) J. Mol. Biol. 31, 155-176

Reedy, M.K., Bahr, G.F., Fischerman, D.A. (1973) Cold Spring Harbor Symp. Quant. Biol. 37, 397-421

Reedy, M.K., Garret, W.E. (1977) In: Insect Flight Muscle (Tregear, R.T. ed.) pp. 115-136, North Holland, Amsterdam

Reedy, M.K., Holmes, K.C., Tregear, R.T. (1965) Nature (London) 207, 1276-1280

Reedy, M.K., Leonard, K.R., Freeman, R., Arad, T. (1981) J. Muscle Res. Cell Motil. 2, 45-61

Slayter, H.S., Lowy, S. (1967) Proc. Nat. Acad. Sci. (USA) 58, 1611-1618

Squire, J.M. (1972) J. Mol. Biol. 72, 125-138

Squire, J.M. (1977) In: Insect Flight Muscle (Tregear, R.T. ed.) pp. 91-114, North Holland, Amsterdam

Squire, J.M. (1981) The Structural Basis of Muscular Contraction, Plenum Press, New York and London

Stewart, M., Kensler, R.W., Levine, R.J.C. (1981) J. Mol. Biol. 153, 781-790

Takahashi, (1978) J. Biochem. 83, 905-908

Taylor, K.A., Reedy, M.C., Cordova, L., Reedy, M.K. (1984) Nature (London) 310, 285-291

Tregear, R.T., Squire, J.M. (1973) J. Mol. Biol. 97, 279-290

Trinick, J.A., Elliot, A. (1979) J. Mol. Biol. 131, 133-136

Trombitás, K., Tigyi-Sebes, A. (1979) Nature (London) 281, 319-320

Trombitás, K., Tigyi-Sebes, A. (1980) Acta Biochim. Biophys. Hung. 15, 156-157

Trombitás, K., Tigyi-Sebes, A., Pallay, G. (1977) In: Insect Flight Muscle (Tregear, R.T. ed.) pp. 53-56, North Holland, Amsterdam

Vibert, P., Craig, R. (1983) J. Mol. Biol. 165, 303-320

Weber, K., Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

Wray, J.S. (1979) Nature (London) 277, 37-40

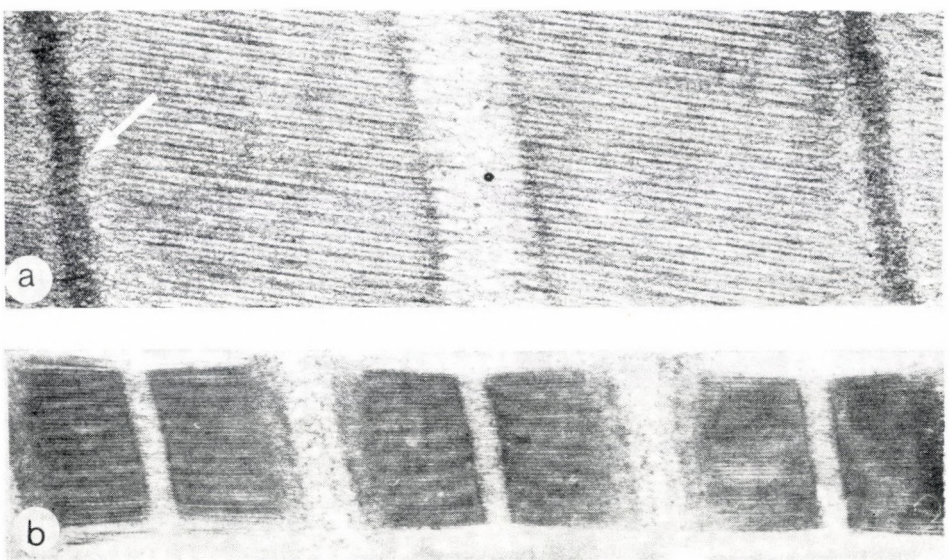
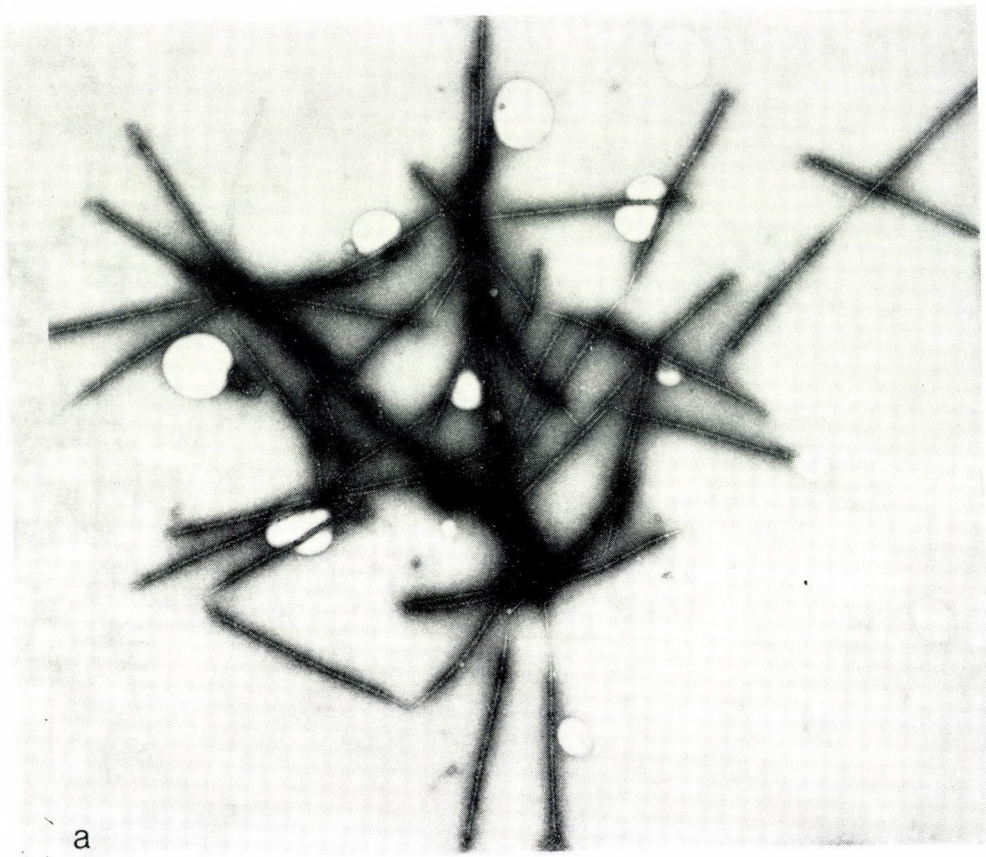
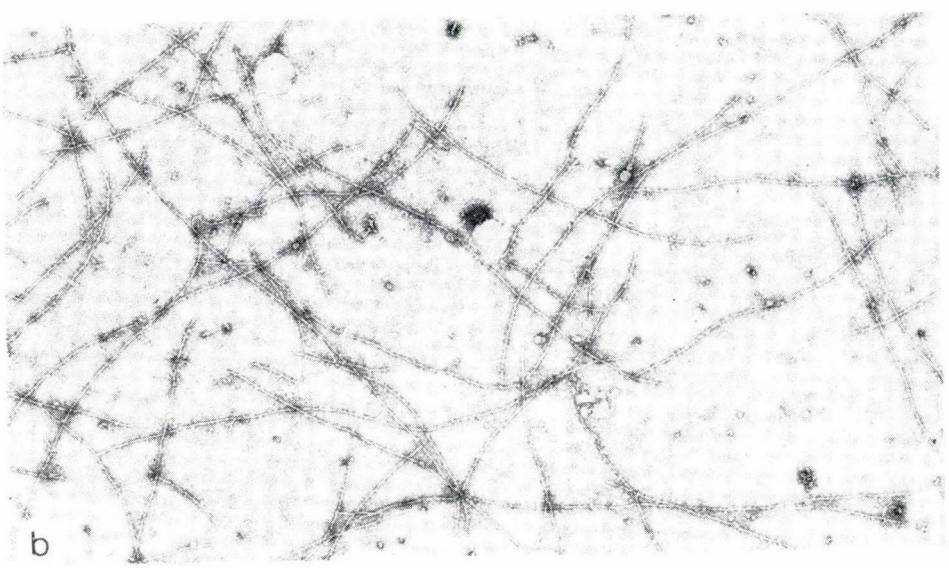


Fig. 1 Conventionally processed insect flight myofibrils for electron microscopy after low ionic strength extraction (see Materials and Methods). *a* The M line material as well as the bare zone of thick filaments were completely and the Z line material partially removed. Note the obliquely running Z filaments (arrow). *b*, Complete removal of the Z line material was produced by the prolonged extraction procedure. The myofibril is divided into half-sarcomeres by the extracted gaps at the M line and the Z line regions. Magnifications: (a) 38,000 × ; (b) 15,000 × .


a


b

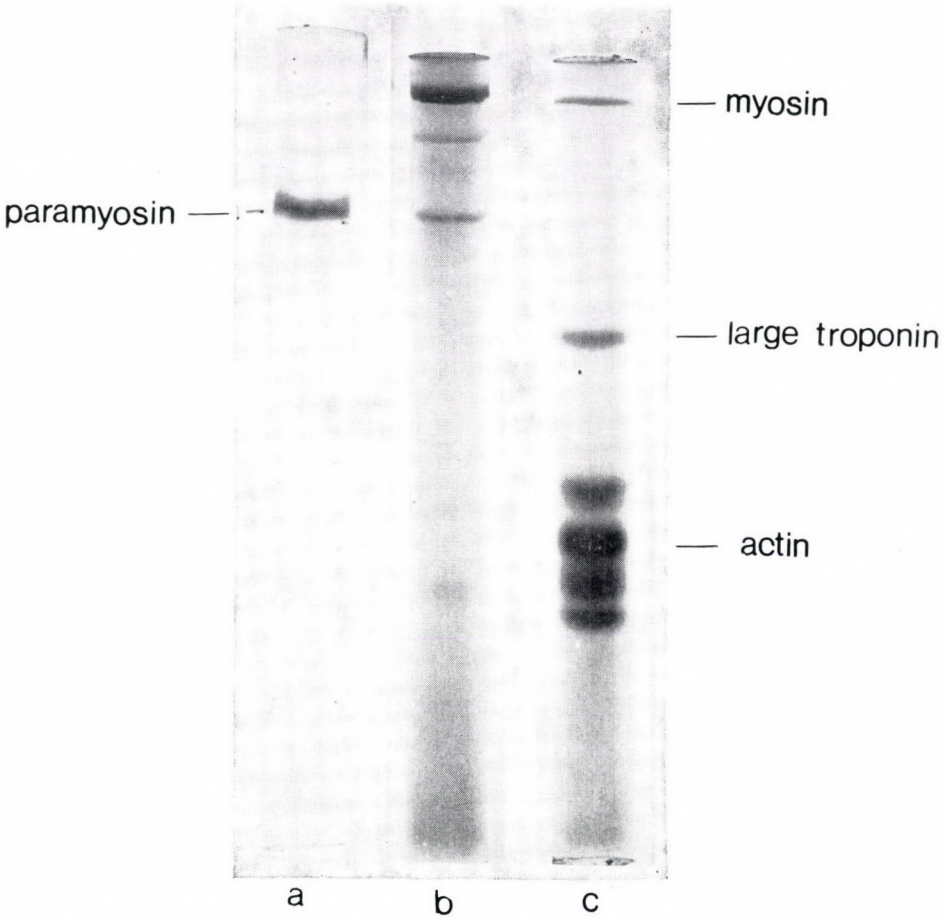


Fig. 3 SDS polyacrylamide gel electrophoresis of purified paramyosin *(q)*,thick filament raction *(b)*, and thin filament fraction *(c)*. Paramyosin was prepared according to the method of Bullard et al. (1973). The paramyosin content of the thick filament is apparent. The thin filaments contain a protein with a molecular weight of about 85,000 identified recent'y as large troponin (TnI; Bullard, 1984).

←

Fig. 2 Negatively stained thick and thin filament fractions separated by zone sedimentation in a density gradient of glycerol. *(a)* Relatively pure thick filament fraction. The filaments are tapered at their central regions which have a flexible property. *(b)* Separated thin filaments. The individual thin filaments are generally longer than a half-sarcomere. Magnifications: *(a)*, *(b)* 28,000 ×.

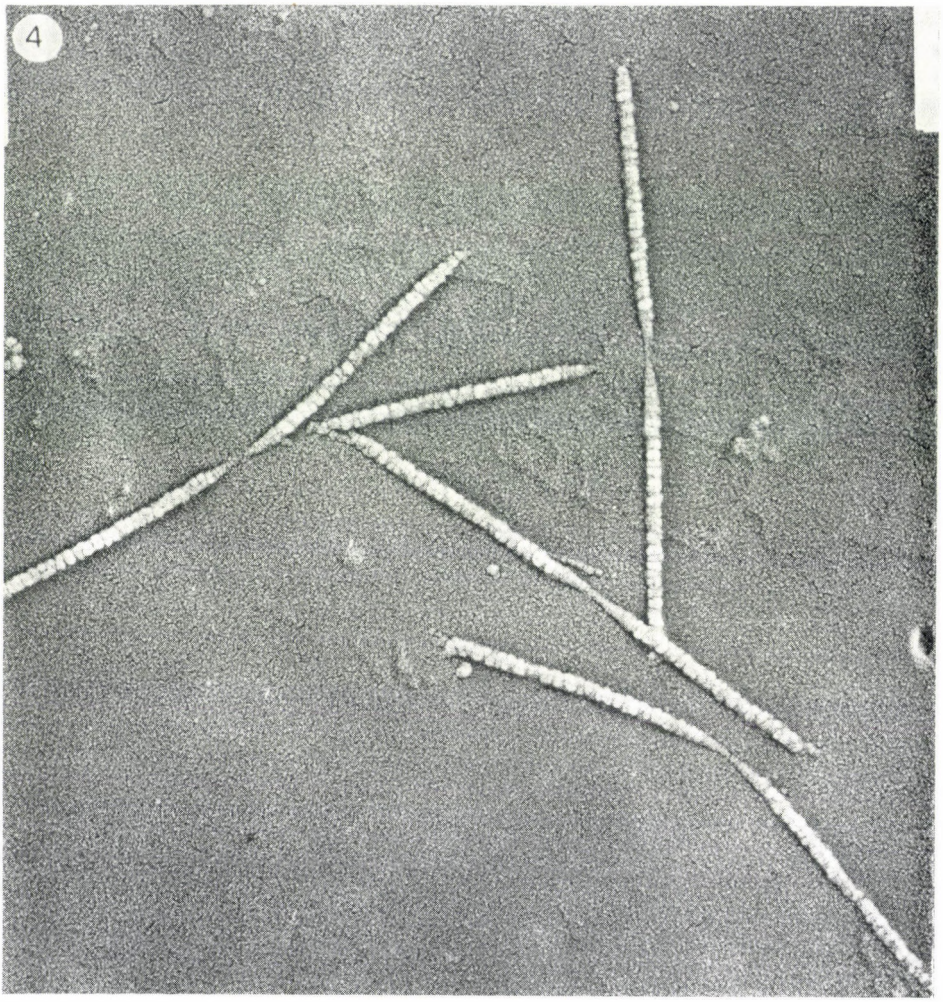


Fig. 4 Rotary shadowed thick filaments with a transverse periodicity of about 38 nm. In half way along the filaments there are tapered flexible zones. Magnification: 30,000 ×.

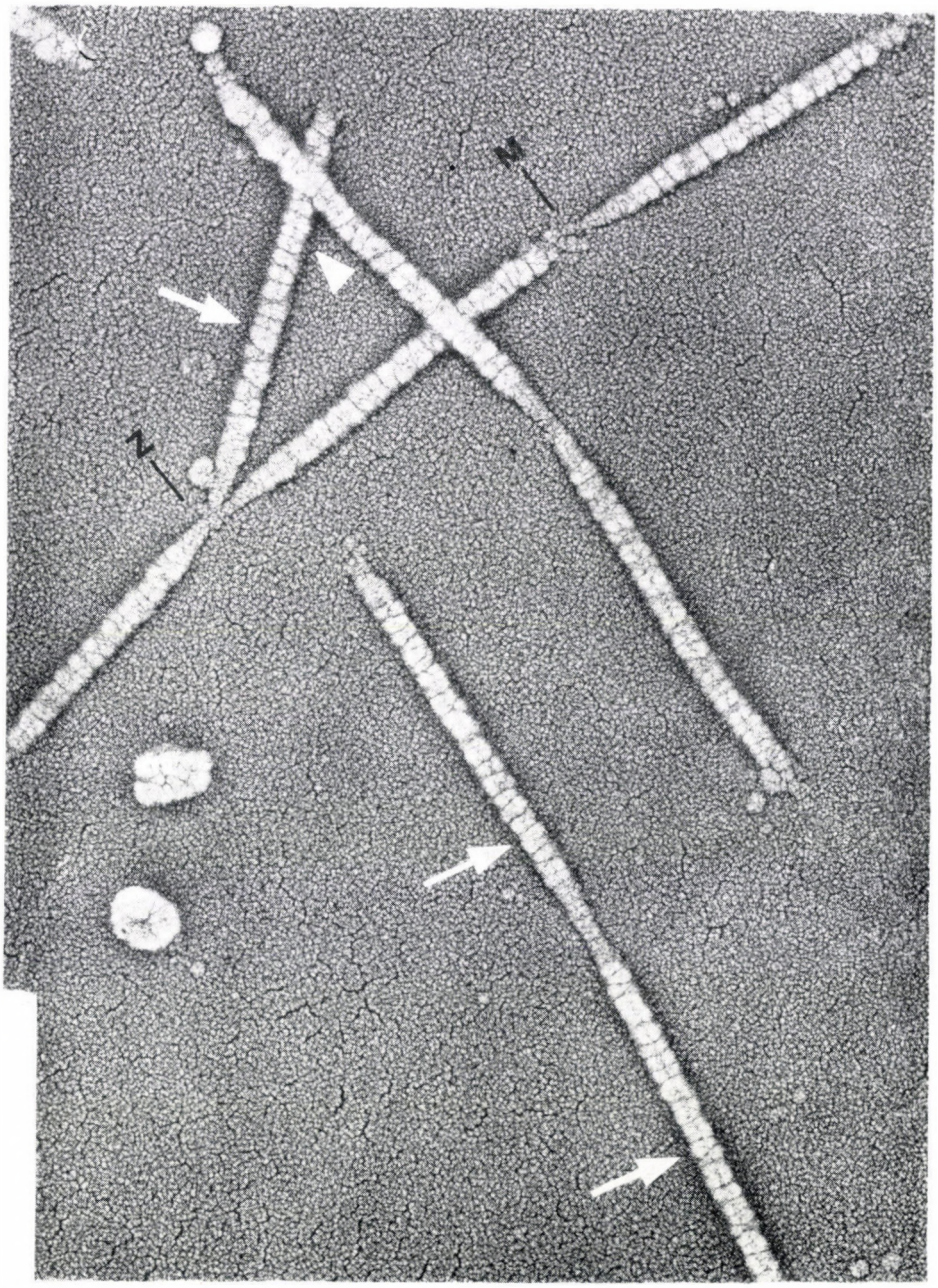


Fig. 5 Rotary shadowed thick filaments at higher magnification. Transverse stripes (arrowhead) and triangular profils (arrows) can be seen on the filament surface. The 3.2 μm long filament on the top of the micrograph is divided into three parts by a bifurcation (Z letter) and an extraction zone (M letter). Magnification: 50,000 $\times$.

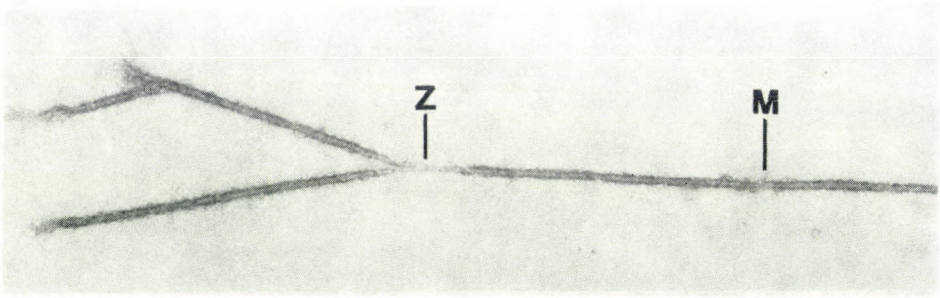
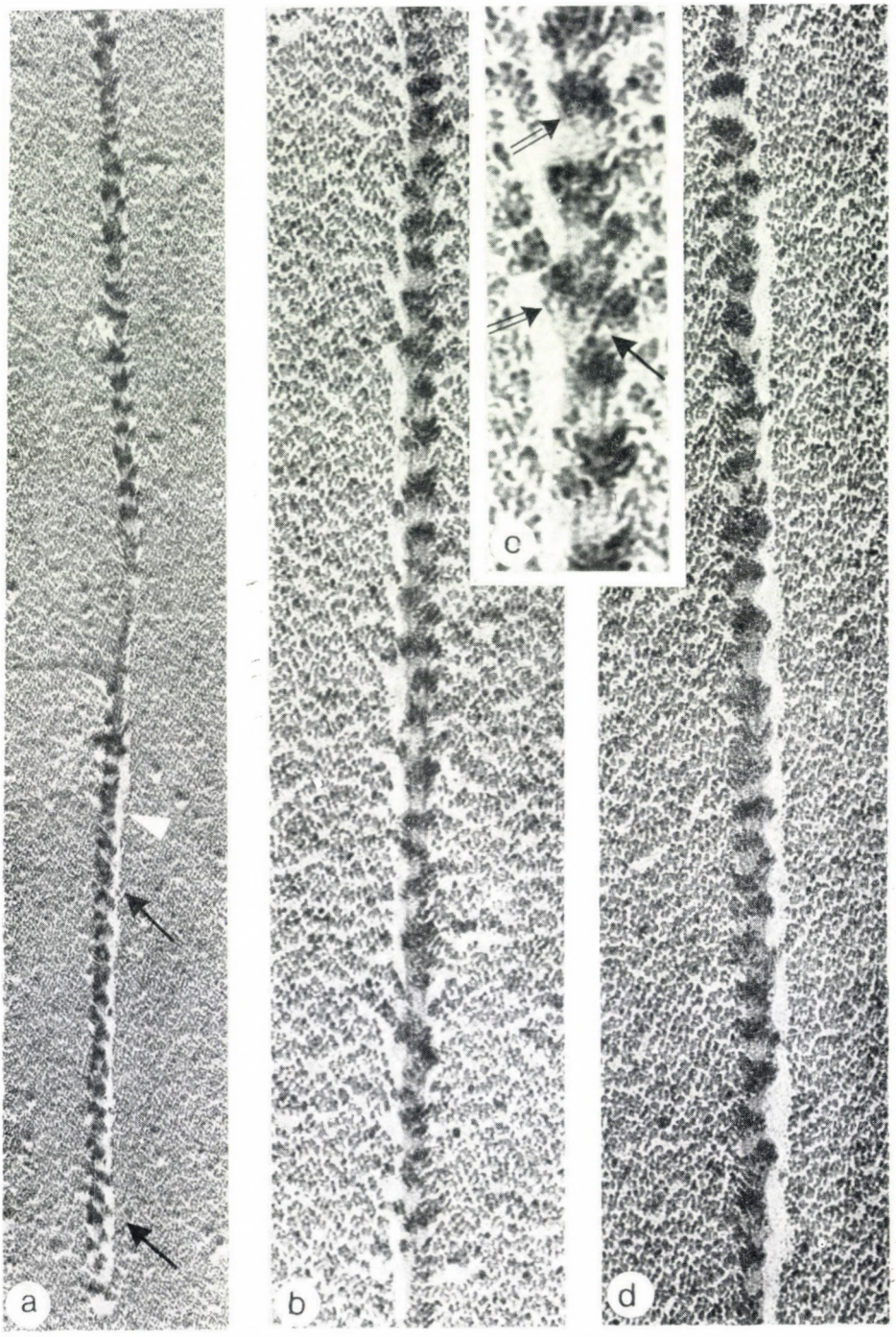


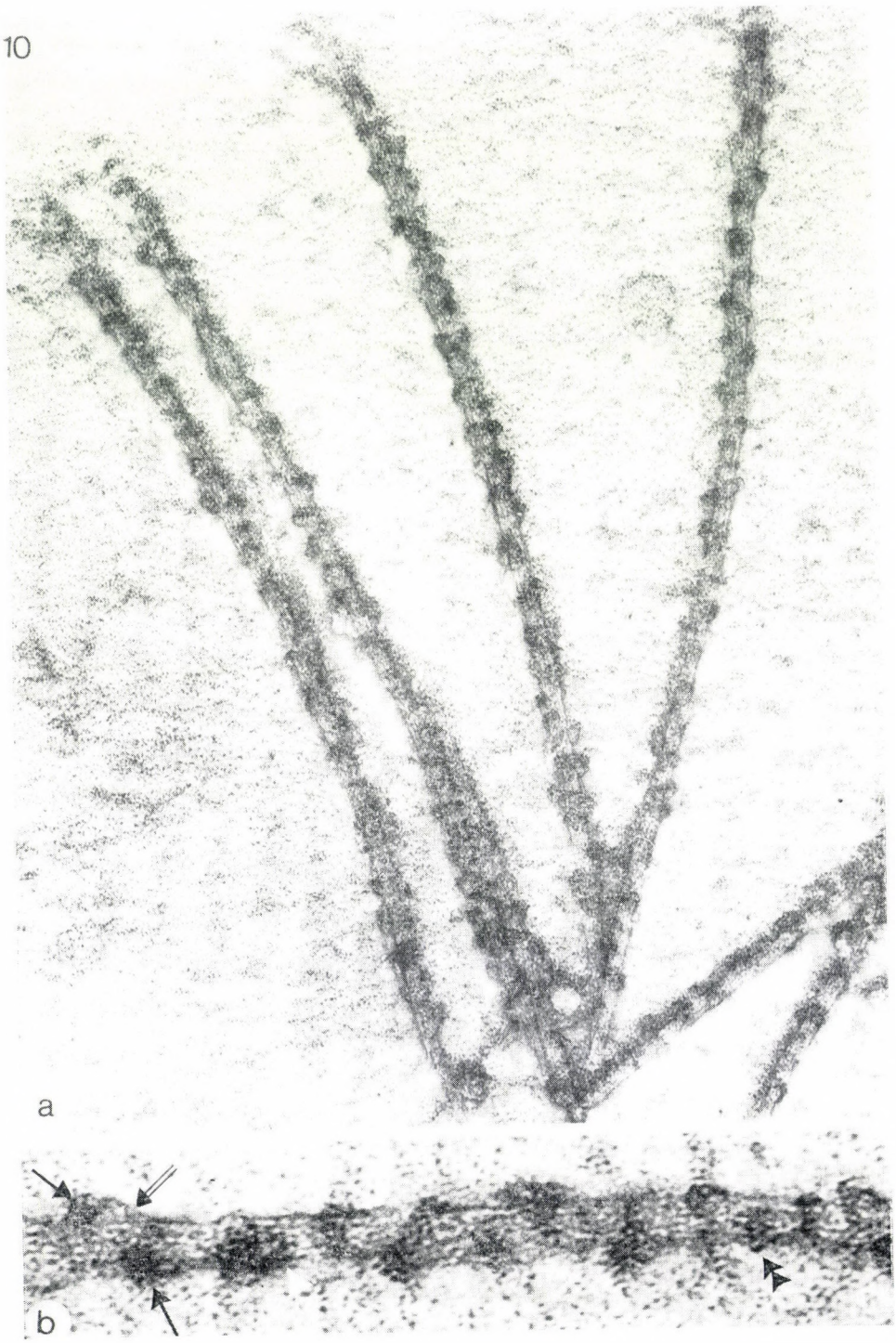
Fig. 6 Negatively stained thick filament with a length of 3.3 μm. The M letter indicates the bare zone, the Z letter the bifurcation zone. Magnification: 38,000 ×.

Fig. 7 Thick filaments lying perpendicular to the direction of shadowing. *(a)*, *(b)* The peaks of projections show a periodicity of about 38 nm with a limited perturbation. *(c)* Similarly shadowed single thin filament shows a 38.5 nm periodicity. Optical shdowing of two-, four- and six-stranded models can be seen on *d*, *3*, and *f* (Reedy, 1968; Wray, 1979; Squire, 1977). Only the two-stranded helices gave the 38 nm shadow peak repeat. Magnifications: *(a)* 70,000 ×; *(b)*, *(c)* 80,000 ×.

a
b
38.5 nm
c
d
e
f


a
b
c
d

10



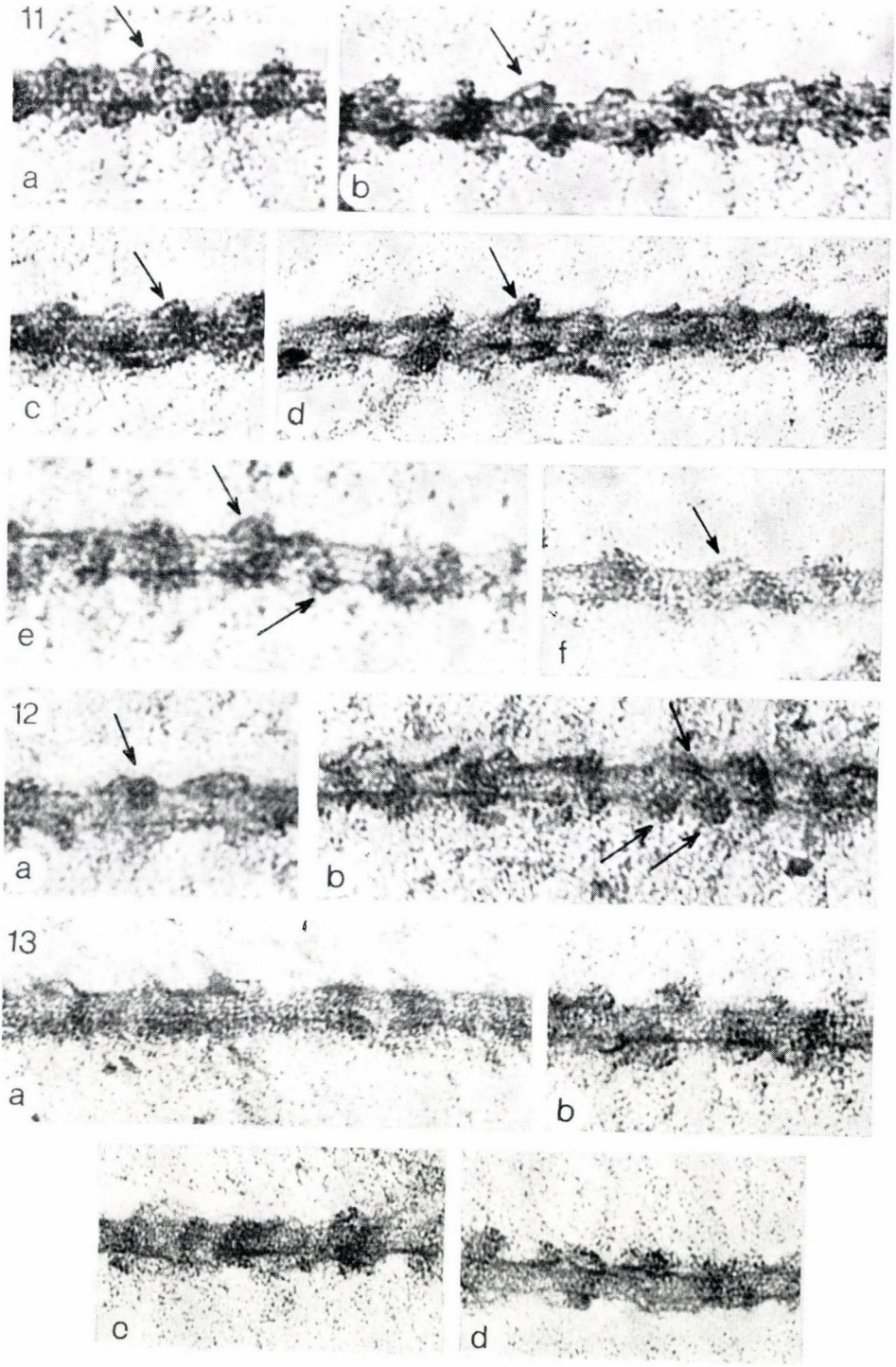

11
a
b
c
d
e
f
12
a
b
13
a
b
c
d

Fig. 8 Thick filaments lying parallel to the direction of shadowing. *(a)* A repeat of about 38 nm is generally produced by the associated projections on the filament surface, but occasionally 14.5 nm repeats can also be seen (white arrowhead). In spite of the association effect of the projections, the left-handed helical surface structure of the filament is observable (black arrows). *(b)*, *(c)* The associated projections show a clear periodicity of about 38 nm. *(c)* The individual projection origins are arranged on a left-handed helical track (black arrow). Pairs of projections appear to originate from a common point (double arrows). Magnifications: *(a)* 75,000 × ; *(b)*, *(d)* 150,000 × ; *(c)* 330,000 × .

Fig. 9 Projection heads can be seen on the surface of a trifurcated filament. Alternate helical gyres can occasionally be observed, which are spaced about 2 × 38 nm apart (white-white and black-black arrows). Magnification: 80,000 ×.

Fig. 10 Thick filaments originated from the prolonged extraction procedure. *(a)* The filament backbone composed of subfilaments. Five subfilaments can generally be distinguished on the filament surface. *(b)* According to the size of the individual projections (black arrows), they are more probably composed of two than a single myosin molecule (double arrowhead). One pair of projections appears to originate from a common point (white arrowhead). The stalk is widened at the origin of the projection as seen in the fringe of the shaft; this might indicate the location of an extra minor protein (double arrow). Magnifications: *(a)* 160,000× *(b)* 425,000× .

Fig. 11 The stalks of the projections are separated from the filaments and turned around the shaft. The individual projection heads are attached to the shaft in different angles, making an impression that the heads of the projections tend to bind to specific sites on the filament shaft (arrows). Magnifications: *(a)*, *(b)*, *(e)* 250,000 × ; *(c)*, *(f)*, *(d)* 190,000 × .

Fig. 12 Arrows point to projections which have two stalks and are composed of more than two myosin heads. Magnifications: *(a)*, *(b)* 250,000 × .

Fig. 13 A fine transverse periodicity of about 4 nm is superimposed onto the subfilaments. Magnifications: *(a)*, *(b)* 250,000 × *(c)*, *(d)* 190,000 × .

Acta Biochim. Biophys. Hung. 21/1-2/, pp.129-154/1986/

ONE-ELECTRON ACTIVATION IN ATP SYNTHESIS

Olga S. Nedelina, Lev P. Kayushin

Institute of Chemical Physics of the USSR Academy of Sciences, Moscow, Soviet Union

(Received February 10, 1984)

INTRODUCTION

The ATP synthesis in oxidative phosphorylation as well as in photophosphorylation, is unique in that the phosphorylating agent in the reaction is orthophosphate (in all other phosphorylation reactions it is the monoester of phosphate: $RO-\overset{OH}{\underset{O}{P}}-OH$). The reaction involves a breakage of the very strong chemical bond, $P-O^-$, which suggests quite hard catalysis. Among model reactions of ATP synthesis, and of the phosphoryl bond synthesis in general, there is only one group that lacks an obvious phosphorylating agent $RO-\overset{OH}{\underset{O}{P}}-OH$, the catalysis in this group is carried out by electrons. This happens in cases of hard gamma and X-irradiation, UV-irradiation, cathode electrochemical synthesis, and syntheses coupled to a redox reaction. It appears that reagents may be rendered highly reactive by way of interaction with electrons.

There is now considerable evidence suggesting the participation of endogenous redox centers in the control of ATP synthesis during oxidative phosphorylation. A high sensitivity of ATP synthetase to a variety of redox agents has also been documented. Based on these facts, we constructed a paramagnetic model of ATP synthesis, in which endogenous redox centers play a direct part in the act of synthesis by providing for electron transport across the ATP synthetase at the moment of energization, and for ADP activation by this electron. The activated, or energized, ADP molecule is conceived to be a semi-reduced

structure, an anion-radical, formed as a result of one--electron transfer.

1. Activation by means of one-electron transfer in nucleophylic substitution reactions and in model ATP syntheses

The discovery of free radicals in the acid-base reaction of ATP hydrolysis by actomyosin dates back to 1961, (Kayushin, et al., 1961). Later Blumenfeld and Tyomkin (1962) proposed a mechanism for this reaction which involved free-radical intermediates.

The mechanisms of synthesis and cleavage of the P-O-P bond are complex and involve elements of acid-alkaline as well as conformational and nucleophilic catalyses (Kirby, 1980). The governing factor is the strength of the bond with the leaving group, and in cases of a weak leaving group thre is a shift towards nucleophilic catalysis as being the most effective in labilization of tight bonds (Kirby, 1979).

The reaction of synthesis and hydrolysis of ATP is classified at present as belonging to the nucleophilic substitution type, S_N2 or S_N1 (Bauerlein, 1978).

Initially, the discovery of free radicals in nucleophilic substitution, reaction was regarded rather as a curiosity. Further studies have, however, shown that intermediate free-radical products are quite common to this type of processes and, as a result, these processes have been classified as a special group of chemical reactions $S_{RN}2$ and $S_{RN}1$ (Kornblum, 1975; Bunnett, 1978; Kim, Bunnett, 1970; Ochlobystin, 1976). These reactions proceed through the formation of intermediate anion-radicals of the nucleophils and the substrate. The whole complex of evidence presently available suggests that the typical nucleophilic reagents in the majority of cases donate only a single electron rather than an electron pair. Reactions of this type proceed under mild conditions and give a high yield of pure products. By contrast to the S_N2 mechanism, they are insensitive to sterical hindrances. An interesting feature of these reactions is that the leaving groups which are not convenient for the S_N1 and S_N2 mechanisms become readily

releasable upon passing to the anion-radical mechanism and are split off at room temperature. This circumstance is very important for the considered reaction of ATP synthesis since phosphorylation here occurs with the breakage of the $P\text{-}O^-$ bond, and O^- is a very weak leaving group. Reactions of this kind are now classified as $S_{RN}1$ and $S_{RN}2$, where R refers to radical and N, as in S_N1 and S_N2, to nucleophilic. The term radical pertains to the nature of the reaction, the term nucleophilic to its result.

The reactions involve the initiation, development and termination stages:

$$[RX]^{\cdot -} \longrightarrow R^{\cdot} + X^-$$

$$R^{\cdot} + y^- \longrightarrow [RY]^{\cdot -}$$

$$[RY]^{\cdot -} + X \longrightarrow RY + [RX]^{\cdot -}$$

The intermediate products are the rather active free radical $R^{\cdot}$ and the anion-radicals $[RX]^{\cdot -}$ and $[RY]^{\cdot -}$.

Summing up of the three stages leads to the following equation:

$$RX + Y^- \longrightarrow RY + X^-$$

Therefore, although the reaction involves free-radical and ion-radical products, its result is nucleophilic substitution. The first and second stages correspond precisely to those of the S_N1 and S_N2 mechanisms, with the exception that the substrate derivatives are one electron richer. The reactions are initiated by generation of any of the radicals mentioned when free electrons are in excess. This happens under illumination (Rossi, Bunnett, 1973), in electrochemical systems (Saveaut, 1980), and in the presence of solvated electrons (Dye, 1968). It is quite natural that typical one-electron transfer agents can also induce a reaction with low activity nucleophils.

The main criterion for classifying a reaction as type $S_{RN}1$ or $S_{RN}2$ is the involvement of free-radicals. Another criterion is the suppression of the reaction by free-radical

inhibitors.

More and more reactions are now classified as $S_{RN}1$ or $S_{RN}2$ type processes. What is particularly important, some reactions involving conversions of phosphorus compounds also belong to this type (Levin, Vorkunova, 1978).

Some of the model reactions of ATP and polyphosphate synthesis seem to proceed according to $S_{RN}1$ or $S_{RN}2$ mechanisms, as they meet the appropriate criteria discussed above. The pyrophosphate bond synthesis was shown to become accelerated after introducing free electrons into the system by hard irradiation (Shima, Utsumi, 1961) and exposure to UV-light (Beck, Orgel, 1965; Lozinova et al., 1981b), when free-radicals of phosphate and nucleotides are formed. Sensibilized ATP synthesis was reported to occur after absorption of visible light by the chromophores, probably with subsequent transfer of the energy to the nucleotide (Goncharova, Evstigneev, 1977). ATP synthesis was also demonstrated during electrolysis of a solution containing ADP and phosphate (Stradyn, Giller, 1975), and pyrophosphate synthesis was observed in a system containing only phosphate (Baudler, Shellenberg, 1968). Electrosynthesis of ATP was achieved in model system (Rögner et al., 1979) and in chloroplasts placed between electrodes (Tsong, 1983).

In an illuminated model system containing ferrochemochrome, imidazole, ADP and phosphate the EPR method revealed production of free-radicals at the stage of the intermediate pentacovalent compound formation (Wang, 1979).

All the above-mentioned reactions proceeded in a non--aqueous medium, i.e. in a medium containing few free protons capable of interacting with free-radicals and thus inhibiting the ATP synthesis. This is another indication in support of the described reaction mechanism.

Electrons needed for activation of the reaction participants, ADP and phosphate, can also be provided by redox processes which often occur in two one-electron stages. This circumstance makes highly likely the formation of an intermediate phosphorylating agent owing precisely to the interaction of the semi-oxidized or semi-reduced product with

phosphate (Bauerlein et al., 1971).

Thus, ATP synthesis can proceed and in some cases actually does proceed in accordance with the $S_{RN}1$ or $S_{RN}2$ mechanisms.

In the case of $S_{RN}1$ mechanism, the P-O^- bond is more easily broken in the anion-radical, i.e. the leaving O^- or OH groups can leave readily (Mishra, Symons, 1973). As it has already been mentioned, the $S_{RN}2$ mechanism involves the formation of an intermediate pentacovalent structure, having the shape of a trigonal bipyramide and characterized by hybridization of orbitals (Kirby, 1978). Of great importance in this case is the ability of an attaching particle to form a bond at a long distance. This occurs more easily if the attaching agent (nucleophil) is a free-radical. Let us assume that the free-radical is ADP which may be considered as a phosphoranyl free-radical (ADPO$^\bullet$) with a spin density located at the oxygen atom. Although the initial electron capture is achieved most likely by the aromatic fragment (bases capture electrons 10^5 times more efficiently than phosphoryl residues) (Suni Samuni, Neta, 1973), the electron is then apparently passed to the terminal phosphorus group. In principle, such a transfer is possible (Wilson, Gulick, 1972) but depends on the nature of the bridge between the aromatic fragment and the phosphorus residue since it determines the angle between the π-system axis and the phosphorus orbitals. A folded configuration, both in ATP and in ADP, should favour this transfer according to the spin-polarization mechanism (Kennard et al., 1971). There are indications that nucleotides may contain free-radicals of phosphorus. The ENDOR method clearly reveals at 6K signals from three free radicals in a CMP monocrystal X-irradiated at 11K, one of them being the oxygen-centered radical in the phosphate fragment (Close, Bernhard, 1979).

The formation of ADP free-radical is always associated with utilization of high energy. The reduction potential of ADP is high enough. Electrochemical data indicate that the one-electron wave which characterizes the anion radical formation occurs at the cathode potential of - 2.5 V (Lozinova

et al., 1981a). The entire respiratory chain of mitochondria functions in the range between -0.250 and +0.8 V (Eresinska et al., 1974) and, at the first glance, contains no compounds capable of reducing ADP. The potential values in the respiratory chain are, however, not invariable and strongly depend on measurement conditions. When exposed to certain factors, such as polarity of environment, formation of a bond with protein, formation complexes or electron excitation, they may vary greatly and do vary as a result of energization (Dutton, Wilson, 1974).

The formation of ADP anion radical upon interaction with flavin semiquinone has already been documented (van de Vorst, 1971, 1973). ATP synthesis was also observed during illumination of a mixture of ADP, $P_{inorg.}$ and flavin at the flavin absorption band (Lozinova et al., 1983). The simultaneously recorded change in ESR absorption was due, most likely, to the appearance of the ADP radical. The proposed model has the advantage of enabling us to analyse the mode of activation of ADP, which is tightly bound at the ATP synthetase catalytic site, as a result of one-electron interaction with the flavin semiquinone which in turn, is also involved at the coupling point. This interaction is thought to markedly increase the affinity of ADP to $P_{inorg.}$, i.e. eventually, to lead to ATP synthesis.

2. Paramagnetic model of ATP synthesis in oxidative phosphorylation based on endogenous redox centers

The free-radical mechanism of coupling that we postulate here involves a number of points which must be met, the impossibility to meet them in a biological system would invalidate all the above considerations. This concerns the formation of the free-radical ADPO$^{\bullet}$, its stabilization, and the removal of the unpaired electron from the system, since accidental attachment of the latter to any other group may lead to devastating consequences for the whole system. In other words, the ATP synthetase must be capable of providing for the uptake, transfer and removal of the electron, i.e. be a redox enzyme

and have its own paramagnetic centers (as far as activation by one-electron transfer is concerned). There is experimental evidence suggesting that this is indeed the case. First of all, we should like to say about the existence of an energy-dependent pool of one-electron transfer agents. In mitochondria apparently only the cytochromes represent a simple linear sequence of devices undergoing redox transformations. The rest of the respiratory chain components, pyridine nucleotides, flavins, non-heme iron, CoQ, are present in much larger quantity than cytochromes and can also be engaged in other functions, besides the direct transfer of electrons to O_2.

A special energy-dependent flavin fraction which can be reduced only in the presence of ATP but not under anaerobic conditions at the I coupling site was discovered by Klingenberg (1966) and then by Chance (Chance, Schöner, 1966) using optical and fluorescent spectroscopy methods. The iron-sulfur N1a, N2 complex is also subject to influence on the part of ATP (Ohnishi, 1979).

The redox centers that are obviously not respiratory transfer agents yet are subject to energization are characteristic not only of the I coupling site but also of the II and III ones.

In this laboratory the existence of a separate electron-transport chain directly coupled to ATP synthesis has been also demonstrated using the EPR method.

2.1. Flow EPR spectrometer for simultaneous recordings of the EPR signal with g = 2.00 and of biochemical characteristics of intact mitochondria

The free-radical EPR signal with g = 2.00 which is observed at room temperature in whole tissue, mitochondria and at various stages of their disintegration, is due to redox processes which involve flavin as a major participant. At the same time, flavins are obviously not the only paramagnetic centers present in the mitochondrial membrane. It will suffice to say that the membrane contains CoQ, cytochromes, and iron-sulfur proteins which incorporate non-heme iron.

Various physical methods such as lowering the temperature or altering the microwave power are used to separate and identify these centers in EPR spectroscopy, when changes in relaxational interaction result in narrowing of EPR absorption bands. In the recent years redox titration with various mediators has been widely used (Beinert, 1972, 1977). All these methods, while being absolutely necessary for characterization of a paramagnetic center, are unfortunately of low value for estimating functional significance of the center, since they require at least some degree of fixation of the biological material.

The most frequently used method of fixation in EPR spectroscopy is freezing. It is generally believed that freezing in liquid nitrogen, particularly by the quick freezing technique, makes the least possible damage to the object while markedly increasing the sensitivity of the method. The technology of low temperature measurements was particularly beneficial for EPR studies of paramagnetic metals contained in biological systems, and it enabled kinetic measurements to be made as well under a variety of conditions.

However, in spite of these advantages, the freezing fixation method inevitably alters the membrane structure, the immediate effect being a change of the redox potential of membrane-located enzymes (Araki, 1977). This circumstance may distort the initial state of the paramagnetic centers and makes very difficult to study their function. In our studies, therefore, the gain in sensitivity and the possibility of kinetic measurements were sacrificed for the sake of investigating the functional role of the free-radical centers, of flavins in particular, which requires EPR measurements of an intact object, with simultaneous monitoring of a number of biochemical characteristics. Since the objects of our study are the mitochondria the problem was aggravated by the existence of the protein concentration barrier. Essentially, it consists in the difference, by more than one order of magnitude, between the concentrations that one is forced to work with in EPR spectroscopy and those concentrations of

mitochondria that were employed in developing their biochemistry. An increase of the so-called physiological concentration of mitochondrial protein, i.e. surpassing the 3 mg/ml level, may often give an entirely unexpected and even opposite result. This is particularly true of the oxidative phosphorylation process and its partial reactions. Therefore, extrapolation of whatever parameters, e.g. rate of oxygen uptake at some metabolic state, to higher protein concentrations is absolutely out of question. In other words, in any investigation concerned with the functional significance of a paramagnetic center, a thorough and adequate control for the maintenance of biochemical parameters should be run.

In particular and in the first place this concerns the supply of oxygen to mitochondria. Being a multienzyme redox system, mitochondria require continuous influx of oxygen for their normal functioning, and the relatively high enzyme concentration in the cell of an EPR spectrometer makes the demanded inflow rather high.

In order to solve these problems, we undertook the development and manufacturing of a special flow system built in the resonator of an EPR spectrometer (Kayushin et al., 1978) This flow system enabled us to maintain mitochondria under their normal biochemical conditions, i.e. supplied with enough oxygen while taking their EPR spectra, which could easily be monitored using a continuous polarographic control with a Clark electrode built into the same system, next to the resonator. The oxygen pressure in the system was maintained at the atmospheric level during the measurements. The set up was constructed so as to allow various reagents to be added to the chamber, in order to realize different metabolic states of mitochondria in the resonator, as well as to take samples for phosphorylation assay. The rapid mixing with the added reagents as well as the high flow rate in the system ensured a quick establishing of a metabolic state in the cell, as it could be judged by changes in the rate of oxygen uptake measured on the polarograph behind the resonator. The adequate conditions for mitochondrium functioning in the resonator

permitted enough accumulation of EPR spectra to be employed for obtaining a good signal-to-noise ratio. In our case the accumulation time was limited only by mechanical damage to the mitochondria that occurred as a result of pumping them through the flow system. Special experiments indicated that such a damage could be neglected in the course of 15 min, a time sufficient for a high signal/noise ratio of EPR spectra.

The combination of the flow system outlined above and an EPR spectrometer allowed us to perform EPR studies of oxidative phosphorylation in mitochondria at a qualitatively advanced technical level and to investigate EPR absorption of mitochondria as a function of a redox state of the respiratory transfer chain and of the activity of mitochondrial ATP synthetase (Brzhewska et al., 1977; Kayushin et al., 1979).

2.2. Energy-dependent EPR signals

In these experiments we confirmed our earlier findings (Brzhewska, Nedelina, 1969) that during coupling, when mitochondria actively synthetize ATP, the concentration of free-radicals in the system is markedly (by 80 per cent) increased as compared to mitochondria supplied only with their oxidation substrate. An uncoupling of oxidation and phosphorylation by DNP eliminates the g = 2.00 EPR signal increase observed during phosphorylation. Inhibition of mitochondria by oligomycin stabilizes the g = 2.00 EPR signal observed in coupled oxidation (Fig. 1). Since oligomycin fixes the enzyme-substrate complex (ADP is at the catalytic site) one is led to believe that the ESR signal, in the presence of oligomycin, is of complex nature and includes signals both from respiratory chain components and, possibly, from the enzyme-substrate complex of ATP synthetase. It should be mentioned in this regard that the g = 2.00 ESR signal of oligomycin-inhibited mitochondria is markedly different from that of DNP-uncoupled mitochondria, in both intensity and shape (Vishnevsky et al., 1980). For better understanding of the nature of the signal, we studied its dynamics as the mitochondria came into contact with the inhibitor. The experiment employed preparations of mitochondria

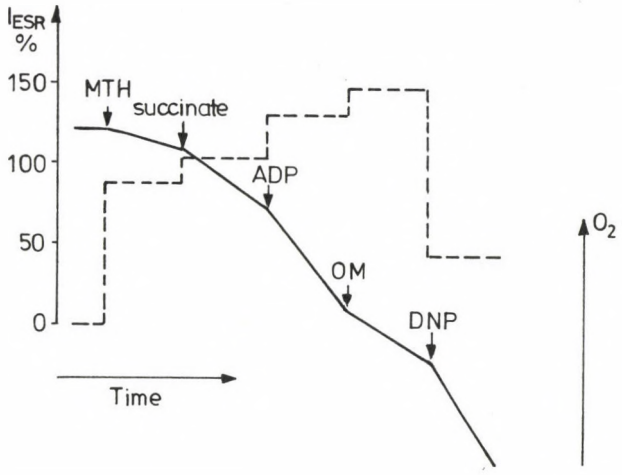


Fig. 1 Dependence of EPR signal with g=2.00 in mitochondria on their oxidative and phosphorylating activities. Mitochondrial suspension was placed in the special flow system in a medium containing 125 mM sucrose, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM $MgSO_4$, 5 mM glucose, 2 mg/ml hexokinase, 10-12 mg per ml mitochondrial protein; additions were: 20 mM succinate, 0.5 mM ADP, 1 µg per 1 mg oligomycin or protein, 0.5 mM 2,4-DNP; EPR conditions: power: 20 mW, modulation amplitude: 6 gauss, modulation frequency: 100 KHz, temperature: 25°C, scanning rate: 300 gauss/min, microwave frequency: 9.2 gHz, time constant: 0.5 sec, record time: 20 min.

frozen at -196°C at different time intervals after adding oligomycin (Nedelina et al., 1980). As it can be seen in Fig. 2, there is a time-related change in the shape of the spectra. The spectra consist of a sum of several ESR signals, the distance between maximum slope points being 0.003 $\pm$ 0.0005 T. A complete interpretation of the spectrum presents

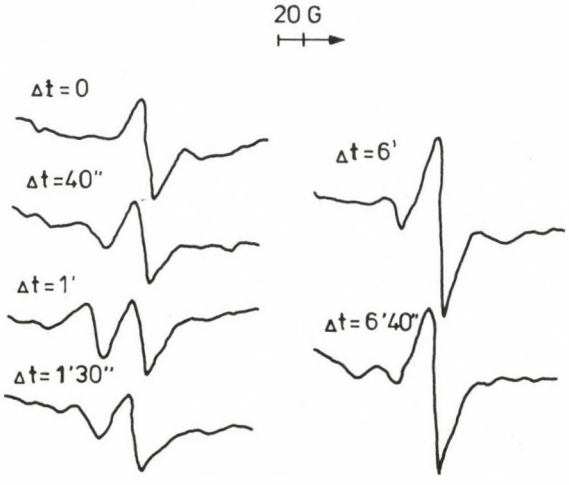


Fig. 2. EPR absorption of mitochondria at different times after oligomycin addition. Conditions were similar as depicted in Fig. 1, except that temperature was 77 K.

considerable difficulty, yet it may be stated that at least three paramagnetic centers, interacting with one another in

time, are responsible for its characteristics. This follows from the fact that the signal components do not change in a uniform manner as the microwave power is varied (Nedelina et al., 1982). Besides the free-radical component at g = 2.00 (of flavin origin), there is also a g = 2.03 component which is not subject to saturation with increasing the power. The latter originates, most likely, from an iron-sulfur complex which plays a direct part in the ATP synthetase function. Also observed is a doublet line with a 0.003 T splitting whose components are saturated synchronously. The doublet line parameters are identical with those of the phosphoranyl radical signal (Ginns, Symons, 1975).

Therefore, an energized mitochondrial membrane, by contrast to the deenergized one undergoes a change in the redox potentials of certain redox centers. Among these centers, the flavin coenzymes and the iron-sulfur centers should be mentioned in the first place. Whether CoQ is involved is subject to a special investigation. The EPR parameters of the redox centers associated with an energized membrane are identical for all the three coupling points, which suggests that the centers characterize not only the respiratory transfer agent, which transforms energy at a certain site of the respiratory chain, but also the coupling point as such, when the membrane is energized.

An additional confirmation for the presence of redox centers in the coupling points is offered by experiments with spin labels: membrane energization is accompanied by reduction of the labels that are specific for ATP synthetase (Azzi et al., 1973).

Therefore, proceeding from the evidence on additional EPR absorption (g = 2.00) of supposedly flavin origin which accompanies energization and phosphorylation at the different levels, in intact mitochondria and in SMP, one comes to the idea of analysing the ATP synthetase itself.

2.3. EPR-absorption of F_1-ATPase

The F_1-ATPase preparations do not show any detectable EPR signal typical of flavosemiquinones. In order to reveal flavins in the enzyme we used a method proposed by Massey and Palmer (McCormick, 1971) for the detection of flavins in flavoproteids. The method is based on the fact that illumination of flavoproteids with visible light causes reduction of the flavin moiety to the semiquinonic state.

When exposed to light at wavelength corresponding to absorption of flavin, F_1-ATPase displays and EPR signal with parameters typical of the flavin group in the protein (Fig. 3) (Nedelina et al., 1983). In the control experiment, after

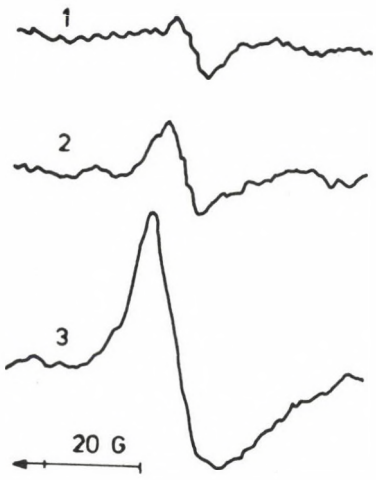


Fig. 3 Effect of visible light on mitochondrial F_1-ATPase (1 mg/ml).
1: dark,
2: illumination $\lambda > 600$ nm:
3: illumination $\lambda > 320$ nm.

illumination with red light, the EPR signal is absent. The amplitude of illumination-induced signal is, to a large extent, a function of the initial redox state of the enzyme. When the enzyme was treated with reducing agents, dithionite or bicarbonate at appropriate concentrations, a significant dark signal was observed, only slightly increased by illumination. However, if the F_1-ATPase was pre-oxidized with oxygen (blowing for 30 min) or with dichlorophenolindophenol, the intensity of a negligible dark signal was stimulated by illumination by 5 to 10-fold.

These data can apparently be regarded as confirmation of the evidence from the literature (Galante et al., 1979) that the mitochondrial ATP synthetase contains a flavin group.

2.4. The relationship between energization of mitochondria and the redox state of flavins as an evidence for direct involvement of flavins in ATP synthesis

A common method used to confirm the involvement of a chemical group in a process consists in a chemical modification of the group that makes it inactive, accompanied by a parallel monitoring of the process in question. The validity of this approach is somewhat lowered by possible side-effects of the modifying agent.

The same principle has been used in our study, in so far as side-effects of modification can be brought to a minimum in the case of flavins. This we owe to the outstanding properties of these unusually flexible and readily interactable molecules. In this particular case we used the flavin's property of becoming reduced when illuminated with visible light.

It may be worth noting that considerable development have been made in the recent years in the branch of photobiology concerned with the effects of visible light on biological organisms normally functioning in the dark (Montiagnolli, 1977; Hug, 1981). The principal target for visible light in biological systems, according to many investigators, is the flavin molecule (Senger, Briggs, 1981). As it was shown by Packer (Aggarwal et al., 1978) the functional degradation of mitochondria exposed to visible light was accompanied by destruction of the flavin molecule. The effect took place after illumination for many hours and was irreversible. However, at early stages of this process only the uncoupling of oxidation and phosphorlyation may be observed, without any disturbance in other functions of the mitochondria. Further illumination leads to growth of the ATPase activity, inhibition of respiration, and fall of the membrane potential to zero, in that order. The P/O ratio and the respiratory control come down at the very beginning of the exposure (although the ability of mitochondria to retain the electrochemical gradient disappears only after a

6-hour illumination), suggesting a direct effect of light upon ATP synthetase or upon the locus that couples it to the electron-transport chain.

We studied (Vishnevsky et al., 1984) the response of mitochondria only to a short-term illumination. The exposure for 30-40 min results in a decrease of the respiratory control together with the EPR signal (Fig. 4). The effect was absent

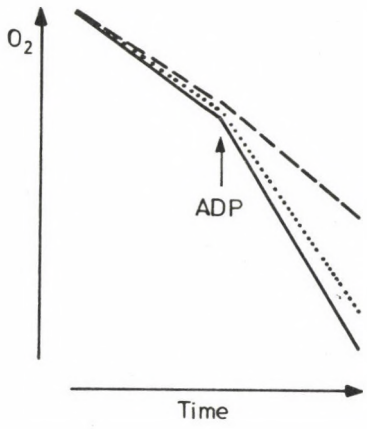


Fig. 4 Effect of visible light and oxygen on coupling activity of mitochondria. Dark: ————, light: - - - - -, light and O_2: ..... . Conditions were the same as depicted in Fig. 1.

when the illumination was carried out in the presence of oxygen (bubbling through the illuminated mixture) or disappeared when oxygenation followed illumination. These results are indicative that the redox state of the flaving group (neither the cytochromes nor CoQ are affected by such illumination) is essential for the oxidative phosphorylation process. Its rate decreases when flavin is completely reduced, while in the presence of oxygen, when the $Fl_{red} + O_2 \longrightarrow Fl_{ox}$ process takes place, the rate is partially restored This makes possible the assumption that the reduced flavin group is inactive, i.e. that the functioning of ATPase-associated flavin proceeds from Fl_{ox} to Fl_{sem} rather than from Fl_{red} to Fl_{sem} like in the case of electron transfer in electron-transferases.

Returning to the properties of the flavin molecule, it should be stressed that, owing to its plasticity, the molecule can play different roles, i.e. while being a selective receptor of environmental influences it may also be able to modify its environment. In other words, this molecule may be a key link

in the process of enzymatic regulation.

The chemistry and biochemistry of flavoproteids has accumulated a number of examples for the regulatory activity of the flavin group. This activity is based on the ability of the molecule to be in three states of oxidation - in the reduced (hydroquinonic form), semireduced (semiquinonic form) and oxidized (quinonic form) states, each of which has a different affinity towards amino acid residues. Thus, the semiquinonic form is distinguished by a higher affinity to tryptophane, tyrosine, serotonin (Lai-Lu, Ingracham, 1976), to substrates and inhibitors, to metal centers (Nishimoto et al., 1980), to the electron and proton (Walsh, 1980), and also distinguished by a specific planarity pattern (Dixon et al., 1979). Clearly, the transition from one of the states to another would bring about microenvironment changes which may be essential for the catalytic act. An interesting phenomenon in this respect presents the so-called "reductive" activation of flavoproteids under exposure to dithionite or visible light, resulting supposedly in a conformational change of the enzyme, which was observed in the case of succinate dehydrogenase and nitrate reductase (Gutman, Silman, 1976; Roldan et al., 1978).

All these results suggest a high sensitivity of flavin to microenvironmental conditions and of its excellent flexibility which ensures an immediate response and thus new, directed changes in the enzyme. One may think that in a number of instances flavin may trigger the appearance of a "combination of electron structures, matching of their chemical properties by way of local electrostatic or conformational influences in order to arrive at optimum values for the given process" (Lichtenshtein, 1979).

Bearing in mind the interrelationship between the apoenzyme protein and flavin one may hypothesize that the disturbances in the ATP synthetase that accompany its energization induce the transition of flavin into the semiquinonic form.

In the proposed scheme for ATP synthesis we proceed from the assumption that ATP synthetase in mitochondria is an enzyme containing several redox elements interacting with one another,

namely, flavin, ADP and, probably, non-heme iron. The reaction of ATP synthesis is provided for by the entry of an electron in the ATP synthetase system from the respiratory chain, the transfer of the electron to ADP to activate it, most likely via flavin with the formation of the semiquinonic intermediate state, and the exit of electron out of the ATPase system back into the respiratory chain. The interaction between the ATPase system elements is made possible owing to the conformational rearrangement of the enzyme that accompanies its energization (Vignais, Satre, 1984; Penefsky, 1978). Such a rearrangement could result in drawing together the redox center of ATPase and its ADP-binding site and the formation of a cluster that would make possible electron transfer.

An example to the above may be offered by the intricate adrenodoxin reductase complex (Lambeth, Kamin, 1980). The rate-limiting step in this reaction is the transfer from the flavin to iron-sulfur center since the slow stage, a conformational change, occurs only after formation of an interenzyme complex. Protonation of an amino acid residue in the vicinity of the redox center changes the redox potential, thereby increasing the electron transfer rate between the flavin and the iron-sulfur centers (Beinert, 1977). In general, it should be stressed that the control of electron transfer rate by means of drawing together redox centers is not uncommon in flavoproteid catalysis.

2.5. ATP synthesis by soluble F_1-ATPase under exposure to visible light as a model for energy-dependent dark coupling

Soluble F_1-ATPase is generally held to be an uncoupled form of an enzyme which can synthesize ATP after energization (Schäfer, 1978). Energization of F_1-ATPase is conceived as a complex change of the molecule under the influence of "energy pressure", the energy being supplied from the respiratory chain of mitochondria. An essential element of this change is conformational rearrangement of both the membrane and the enzyme (Kazachara, Penefsky, 1977). Our model suggests that this conformational rearrangement should draw together the

flavin and ADP-binding sites and cause a change of the redox potential of the system to make it able to accept an electron from the respiratory chain and thus enable the flavin to undergo transition into a semiquinonic form which is capable of reducing ADP to anion-radical (Nedelina et al., 1981).

As far as we deal with a chromophore in the enzyme molecule, it is natural to attempt to simulate its functioning, how it takes place in the dark in vivo, and under illumination.

Photochemical reactions can be viewed as a means of activating compounds that have low reactivity in their usual state. For a transition into an excited, highly reactive state such compounds would need energy. This energy is provided by light. Excitation under the influence of light is a classical example of transition into the excited state.

A chromophore bound to a protein or to membrane absorbs a quantum of light. The enzyme, energized by the light, relaxes to its basic state via several intermediate excited states. These relaxational transitions entail both dynamic and static conformational transitions in the protein of the membrane to which the chromophore is bound. Such a coupled relaxation may affect the membrane potential and the structural specificity of the chromophore relative to its locus on the protein. Light-induced excitation can alter the pK of both acidic and basic groups of the chromophore. Besides the obvious changes in the proton flow and the transmembrane potential, light induces marked changes in the pK of the functional group of proteins, which may also cause static and dynamic conformational changes in the protein membrane. During the existence of the excitation states induced by light, eximers and exiplexes may be produced, capable of overcoming activation energy barriers (Galstone, 1977).

The facts presented above suggest a conclusion that light can induce a transition of the protein molecule of an enzyme into a state which simulates its properties and reactivity in the course of enzymatic reaction occurring in the dark (Cilento, 1980). A similar situation may be supposed to take place with the mitochondrial ATP synthetase. By exposing its chromophores (i.e. flavins) to light, we can hope to transfer

it into an excited, energized state capable of synthesizing ATP, thereby simulating energization which takes place in oxidative phosphorylation. The catalytic effect of light in this case consists in photoreduction of flavin, and this may be followed by a dark reaction between the flavin semiquinone and ADP.

An attempt to obtain light-stimulated ATP synthesis with the isolated F_1-ATPase of chloroplasts has already been reported (Deway, Hammes, 1981). We undertook experiments aimed at obtaining a similar light-dependent ATP synthesis with mitochondrial F_1-ATPase (Nedelina, et al., 1983).

When a mixture of ADP and inorganic phosphate solutions was illuminated with visible light at room temperature in the presence of soluble F_1-ATPase for 15 min, the yield of ATP obtained, paralleled by an appropriate decrease in ADP, was 37.5 nmol/mg protein for a crude enzyme preparation and 60 nmol/mg protein for an enzyme preparation purified from low molecular weight contaminating compounds (adenylate kinase etc.) (Pejefsky, 1977).

3. True uncouplers as inhibitors of one-electron interaction between flavin and ADP

The proposed model can offer explanation not only for the respiratory control phenomenon, i.e. stimulation of respiration after addition of phosphate acceptor, ADP, but also for the stimulation of respiration caused by the so-called true uncouplers.

Considering ATP synthetase to be a redox unit which becomes included in the respiratory chain only in case of energization of the membrane, one may hypothesize that it serves as a shunt to provide electron current necessary for interaction between neighbouring respiratory transfer agents (enhancement of electron transport or of respiration during phosphorylation) as well as to ensure one-electron activation of ADP.

The effect of true uncouplers can be explained by assuming that they are incorporated into the same chain that

accomplishes electron transfer through ATP synthetase instead of ADP and provide for the same or even slightly higher rate of electron transfer.

With the realization that the true uncoupler effect is due not to a single property of these substances, but rather to several of them, such as lipophility, donor-acceptor features, steric configuration (Tedeschi, 1981; Terada, 1981; Haustein, 1976), we shall try to demonstrate that the uncoupling effect may be related to an interference of the uncouplers with the electron transfer through ATP synthetase.

The acidic and lipophilic properties can promote accumulation of uncouplers in the membrane, but the mechanism of the effect should, it appears, be based on the redox properties of the uncoupler and of the ATP synthetase components.

The effect of compounds belonging to the phenol class, which are also effective uncouplers, on mitochondrial ATPase has been shown to be similar to their effect on some dehydrogenases, phosphokinase, catalase, and acid phosphatase (Stockdale, Selwin, 1976). These enzymes were inhibited at about the same concentrations as was ATPase. Phenols bind to the sites of enzymes that are normally responsible for binding an adenine fragment of a substrate or cofactor.

Let us now consider the effect of uncouplers on redox processes in view of donor-acceptor properties of these compounds, as well as the ensuing possible interactions with ATP synthetase's redox components and with the electron transport as a whole. It is significant in this respect that the largest group of uncouplers are phenols, i.e. aromatic compounds with conjugated linkages and a system of mobile, delocalized electrons.

In a study by Tollenaere (1973) it was demonstrated that the uncoupling activity of 23 phenols in mitochondria correlated not only with their lipophility but also with their electron--donor/acceptor properties. As the acceptor properties became more expressed the uncoupling activity increased proportionally.

It is well-known that phenols, particularly nitrophenols, being classical uncouplers are, at the same time, effective inhibitors of free-radical reactions, so that their inhibiting action is often used as a criterion for classifying a reaction as $S_{RN}1$ or $S_{RN}2$. The inhibitory effect of phenols is due to their easy reduction and transition into a free-radical state as a result of one-electron transfer both in aprotonic and aqueous media. Radical derivatives of DNP can be generated electrochemically and by chemical reduction with Ti (Borg, 1972; Bilkis, Shteingarz, 1982).

At the same time, the role of uncouplers can be played by compounds possessing a free electron, the so-called spin labels which can easily donate or accept electron, and the uncoupling effect is paralleled with a drop in spin label concentration as measured by the EPR method. This fact suggests that spin labels are directly involved in the redox process. A decrease in the concentration of DNP-based spin label during the uncoupling of oxidative phosphorylation was observed by Hsia (Zimmer, 1977; Hsia et al., 1972). A stable free-radical of DPhPG, used as uncoupler by Coleman (1973) accelerated respiration and inhibited ATP production at the concentration of 10^{-7}M.

With regard to the mechanism of uncoupling, we are particularly interested in studies concerned with interaction of uncoupling agents, phenols and indols, with flavin (Tollin, 1967, 1968). It has been shown that phenols form a complex with flavin in the oxidized, semireduced or reduced states, and the photobleaching i.e. reduction of flavins is inhibited in such complexes. Especially important is the fact that phenols and indols may inhibit light-induced formation of flavin free-radicals (Lozinova et al., 1983; Pereira, Tollin, 1967). In the ATP synthetase system, the same would result in an interception of the electron, coming from flavosemiquinone and intended to activate ADP, by the uncoupler, blocking naturally the process of ATP synthesis. A support to this assumption comes from our experiments in which uncoupling in the presence of phenol was accompanied by a loss in intensity of the free-

-radical EPR signal in the mitochondria. This result, therefore, can be regarded as an additional evidence in favour of the proposed model for the ATP synthetase catalyzed ATP synthesis involving flavin.

CONCLUSIONS

In this paper we made an attempt to demonstrate that there is now sufficient experimental evidence for constructing a model of one-electron activation in the course of ATP synthesis in oxidative phosphorylation.

The basic assumption of the model is the existence of a direct and reciprocal relationship between the high-energy electron and the formation of the macroergic bond of ATP in the sense implied by Szent-Györgyi when he wrote that "in order for energy P to be usable it should become transformed into the electron energy $\sim \rightleftharpoons S$, the reaction being reversible" (Szent-Györgyi, 1968).

In the oxidative phosphorylation system, a completely material significance can be attached to these words. Indeed, and this can easily be interpreted in terms of classical chemistry, the electron energy is the energy of the highly active free-radical, ADPO$^{\bullet}$, and it is precisely this radical the formation of which enables the hitherto inactive ADP to interact with inorganic phosphate, i.e. to produce ATP.

According to our model, the essence of oxidative phosphorylation is the delivery of this electron exactly on the place of its destination, that is to the ADP molecule fixed at the active site of the ATP synthetase. The model implies that the coupling site has its own redox chain for electron transfer. The immediate precursor of ADP in this electron transfer chain is assumed to be flavin. Actually, that an active form of this compound, the flavin semiquinone, partakes in ATP synthesis in oxidative phosphorylation was the finding itself which prompted the development of the model.

This point of view finds support both in the purely chemical and in the enzymatic models, in which light induces formation of the flavin free-radical which sensibilizes the

formation of ADP free-radical, the process being coupled to ATP synthesis.

The validity of our model for one-electron activation of ADP in oxidative phosphorylation is also supported by the numerous examples of chemical synthesis of ATP by systems rich in free electrons.

We hope that this work will serve as a modest illustration, among others, to the words of Albert Szent-Györgyi who was the first to apply quantum-mechanics in approaching biological problems:

"I am deeply convinced that we shall never be able to understand the essence of life if we remain at the molecular level... From whatever side we approach biology, from the side of matter or from the side of energy, we shall inevitably come to electrons."

REFERENCES

Aggarwal, B., Quintanilha, A.T., Cammack, R., Packer, L. (1978) Biochim. Biophys. Acta 502, 367

Araki, T. (1977) Criobiology 14, 144

Azzi, A., Bragallin, M., Tamburro, A., Santato, M. (1973) J. Biol. Chem. 248, 5520

Baudler, M., Shellenberg, D. (1968) Z. Anorg. Allg. Chem. 356, 140

Bauerlein, E. (1978) In Schafer, G., Klingenberg, M. (eds.) Energy Conservation in Biological Membranes. Springer Verlag, Berlin, p. 237

Bauerlein, E., Klingenberg, M., Wieland, T. (1971) Eur. J. Biochim. 24, 306

Beck, A., Orgel, L. (1965) Nature 208, 1000

Beinert, H. (1972) in Swartz, H.H., Bolton, J.R., Borg, D.C. (eds.) Biological Applications of Electron Spin Resonance. Wiley-Interscience, New York, p. 351

Beinert, H. (1977a) in Lovenberg, W. (ed.) Iron-Sulfur Proteins. Academic Press, New York, Vol. 3, p. 61

Beinert, H. (1977b) in van Dam, K., van Gelder, B.F., (eds.) Structure and Function of Energy Transducing Membranes. Elsevier, Amsterdam, p. 11

Bilkis, I.I., Shteingarz, V.D. (1982) J. Org. Chem. 18, 359

Blumenfeld, L.A., Tyomkin, M.I. (1962) Biophysica 7, 731

Borg, D.C. (1972) in Swartz, H.H., Bolton, J.R., Borg, D.C. (eds.) Biological Applications of Electron Spin Resonance. Wiley-Interscience, New York, p. 265

Brzhewska, O.N., Nedelina, O.S. (1969) Biophysica 14, 477

Brzhewska, O.N., Nedelina, O.S., Sheksheev, E.M., Kayushin, L. P. (1977) Dokl. Acad. Nauk USSR 232, 221

Bunnett, J. (1978) Accounts Chem. Res. 11, 413

Chance, B., Schöner, B. (1966) in Slater, E.C. (ed.) Flavins and Flavoproteins. Elsevier, Amsterdam, p. 519

Cilento, G. (1980) Photochem. Photobiol. Rev. 5, 199

Close, D.M. Bernhard, W.A. (1979) J. Chem. Phys. 70, 210

Coleman, P.S. (1973) Biochim. Biophys. Acta 305, 179

Deway, T.G., Hammer, G. (1981) J. Biol. Chem. 256, 1981

Dixon, D.A., Lindner, D.L., Brauchaut, B., Lipscomb, W.N. (1979) Biochemistry 18, 5770

Dutton, P.L., Wilson, D.F. (1974) Biochim. Biophys. Acta 346, 165

Dye, J.B. (1968) Accounts Chem. Res. 1, 308

Erecinska, M., Veech, R.L., Wilson, D.F. (1974) Arch. Biochem. Biophys. 160, 412

Galante, I., Wong, S., Hatefi, Y. (1979) J. Biol. Chem. 254, 12372

Galstone, A. (1977) Photochem. Photobiol. 25, 503

Ginns, I.S., Symons, M. (1975) J. Chem. Soc. Dalton Trans. 4, 515

Goncharova, N.V., Evstigneev, V.B. (1977) Biochimia 42, 963

Gutman, M., Silman, N. (1976) in Singer, T. (ed.) Flavins and Flavoproteins. Elsevier, Amsterdam, p. 537

Hanstein, W. (1976) Biochim. Biophys. Acta 456, 129

Hsia, J.S., Chen, W.L., Wong, L., Long, R., Kalow, W. (1972) Biochem. Biophys. Res. Comm. 48, 1273

Hug, D.H. (1981) Photochem. Photobiol. Rev. 6, 87

Kasachara, M., Penefsky, H. (1977) in van Dam, K., van Gelder, B. (eds.) Structure and Function of Energy Transducing Membranes. Elsevier, Amsterdam, p. 275

Kayushin, L.P., Kofman, E.B., Golubev, I.N., Lvov, K.M., Pulatova, M.K. (1961) Biophysica 6, 20

Kayushin, L.P., Brzhewska, O.N., Nedelina, O.S., Sheksheev, E.M. (1978) Biophysica 23, 1024

Kayushin, L.P., Brzhewska, O.N., Nedelina, O.S., Sheksheev, E.M. (1979) Biophysica 24, 248

Kim, J.K., Bunnett, J.F. (1970) J. Am. Chem. Soc. 92, 7463

Kirby, A.J. (1978) in Porter, R., Fitzsimons, D. (eds.) Phosphorus in the Environment. Elsevier, Amsterdam, p. 117

Kirby, A.J. (1979) in Pullman, B. (ed.) Catalysis in Chemistry and Biochemistry. Dr. Reidel Publishing Company, Dordrecht, p. 25

Kirby, A.J. (1980) in Stec, W.C. (ed.) Phosphorus Chemistry Directed Towards Biology. Pergamon Press, New York, p. 80

Klingenberg, M. (1966) in Slater, E.C. (ed.) Flavins and Flavoproteins, Elsevier, Amsterdam, p. 319

Kounard, O., Isaacs, N., Motherwell, W.D.S., Coppola, J.C., Warmflor, D.L., Larson, A.C., Watson, D.G. (1971) Proc. Roy. Soc. Lond. 325, 401

Kornblum, N. (1975) Angew. Chem. 14, 734

Lai-Lu Lee Yeh, Ingracham, L. (1976) in Singer, T. (ed.) Flavins and Flavoproteins. Japan Sci. Soc. Press. Tokyo-University Park Press, Baltimore, p. 641

Levin, L.A., Vorkunova, E.I. (1978) Homolytic Chemistry of Phosphorus. Nauka, Moscow

Lichtenshtein, G.I. (1979) Multinuclear Redox Enzymes. Nauka, Moscow

Lozinova, T.A., Sokol, O.G., Nedelina, O.S., Brzhewska, O.N., Kazakova, V.M., Kayushin, L.P. (1981a) Vsesoyuznuy Symp. "Magnetic Resonance in Biology and Medicine", p. 25

Lozinova, T.A., Brzhewska, O.N., Nedelina, O.S., Kayushin, L.P. (1981b) Biophysica 26, 394

Lozinova, T.A., Diomina, O.V., Nedelina, O.S., Brzhewska, O.N., Kayushin, L.P. (1983) Biophysica 28, 555

McCormick, D. (1971) in Kamin, M. (ed.) Flavins and Flavoproteins. University Park Press, Baltimore, p. 154

Mishra, S.P., Symons, M.C.R. (1973) Tetr. Lett. 4061

Montiagnolli, G. (1977) Photochem. Photobiol. 26, 679

Nedelina, O.S., Vishnevsky, E.S., Brzhewska, O.N., Sheksheev, E.M., Kayushin, L.P. (1980) Dokl. Acad. Nauk USSR 252, 1497

Nedelina, O.S., Brzhewska, O.N., Kayushin, L.P. (1981) Free Radical Mechanism of ATP Synthesis in Oxidative Phosphorilation. Preprint VINITI, Moscow

Nedelina, O.S., Vishnevsky, E.S., Brzhewska, O.H., Sheksheev, E.M. (1982) Biophysica 27, 483

Nedelina, O.S., Brzhewska, O.N., Piskunov, M.A., Kayushin, L.P. (1983) Biophysica 28

Nishimoto, K., Watanabe, Y., Yagi, K. (1980) in Yagi, K., Yamano, T. (eds.) Flavins and Flavoproteins. Japan Sci. Soc. Press, Tokyo-University Park Press, Baltimore, p. 493

Ochlobystin, O.Y. (1976) in Mechanisms of Heterolytic Reactions Nauka. Nauka, Moscow, p. 21

Ohnishi, T. (1979) in Capaldi, R.A. (ed.) Membrane Proteins in Energy Transduction. Marcel Dekker, New York, p. 1

Pereira, J.F., Tollin, G. (1967) Biochim. Biophys. Acta 143, 79

Penefsky, H. (1977) J. Biol. Chem. 252, 2891

Penefsky, H. (1978) Adv. Eur. 49, 223

Rogner, M., Ohno, K., Hamamoto, T., Sone, N., Kagawa, Y. (1979) Biochem. Biophys. Res. Comm. 91, 362

Roldau, J.M., Galero, F., Aparicio, P.J. (1978) Z. Pflanzenphysiol. 90, 467

Rossi, R., Bunnett, J. (1973) J. Org. Chem. 38, 1407

Saveant, J.M. (1980) Accounts Chem. Res. 13, 323

Schäfer, G. (1978) in Schäfer, G., Klingenberg, M. (eds.) Energy Conservation in Biological Membranes. Springer Verlag, Berlin, p. 243

Senger, H., Briggs, W.R. (1981) Photochem. Photobiol. Rev. 6, 1

Shima, M., Utsumi, S. (1961) J. Inorg. Nucl. Chem. 20, 177

Stockdale, M., Selwin, M. (1976) Eur. J. Biochem. 66, 443

Stradyw, L.P., Giller, S.A. (1975) in Electrosynthesis and Bioelectrochemistry, Nauka, Moscow

Suni Samuni, A., Neta, P. (1973) J. Phys. Chem. 77, 2425

Szent-Györgyi, A. (1968) Bioelectronics. Academic Press, New York

Tedeschi, H.D. (1981) Biochim. Biophys. Acta 639, 157

Terada, H. (1981) Biochim. Biophys. Acta 639, 225

Tollenaere, J.P. (1973) J. Med. Chem. 16, 791

Tollin, G. (1967) in Slater, E. (ed.) Flavins and Flavoproteins, Elsevier, Amsterdam, p. 11

Tollin, G. (1968) in Pullman, B. (ed.) Molecular Co-Associations in Biology. Academic Press, New York, p. 393

Tsong, T.Y. (1983) Bioscience Rep. 3, 487

Vignais, P.V., Satre, M. (1984) Mol. Cell. Biochem. 60, 33

Vishnevsky, E.S., Nedelina, O.S., Brzhewska, O.N., Sheksheev, E.M., Kayushin, L.P. (1980) Biophysica 25, 740

Vishnevsky, E.S., Brzhewska, O.N., Lozinova, T.A., Nedelina, O.S. Kayushin, O.P. (1984) Biophysica 30,

van de Vorst, A. (1971) Photochem. Photobiol. 13, 321

van de Vorst, A. (1973) in Duchesne, J. (ed.) Physico-Chemical Properties of Nucleic Acids, Academic Press, London-New York

Walsh, C. (1980) Accounts Chem. Res. 13, 148

Wang, J.H. (1979) Meth. Eur. 55, 536

Wilson, M., Gulick, W.jr. (1972) J. Am. Chem. Soc. 94, 29

Zimmer, G. (1977) Arch. Biochem. Biophys. 181, 26

BOOK REVIEWS

RECENT PROGRESS IN POLYAMINE RESEARCH. Edited by L. Selmeci, M.E. Brosnan and N. Seiler. Akadémiai Kiadó, H-1363 Budapest, P.O.B. 24, Hungary. 1985. 634 pages.

Polyamines, the small family of amines, has a unique broad scope of biological and pathophysiological function in all living organisms. They play an important role in cellular differentiation and ontogenesis, in tumor promotion and progression, as well as in protein synthesis and regulation of different metabolic processes.

A series of 59 original papers and reviews, excellent contributions to polyamine research are published in this volume, based on the presentations at the International Conference on Polyamines, Budapest, 1984.

The major sections are: Genetic and posttranslational regulation of ornithine decarboxylase and related enzymes - Regulation of polyamine levels and control functions of the polyamines - Enzymes and inhibitors of polyamine biosynthesis - Enzymes and inhibitors of polyamine catabolism - Polyamines in cellular differentiation and tissue regeneration - Significance of the polyamines in cancerogenesis and clinical oncology - Polyamine metabolism in bacteria - Role of polyamines in plant physiology - Macromolecular polyamine derivatives - Methods in polyamine research.

The book can help everyone who takes active part in polyamine research, as well as scientists who seek information about this rapidly extending field.

Susanne Bardócz

PRINCIPLES OF ENERGETICS by K.S. Spiegler, Springer-Verlag, Berlin-Heidelberg-New York-Tokyo, 1983. 168 pages with 21 Figures

The book contains the next of a one-semester course for students of engineering at the University of Berkeley. Due to some interdisciplinary aspects it may be of interest for physico-chemists and biophysicists as well. After the quite general title promising a wider spectrum the reader becomes disappointed because the book deals essentially only with linear processes in a quite restricted way using a mixed (and therefore at first confusing) nomenclature. The treatment places in its focus the comparison of the rate of exergy dissipation and that of entropy production. After the general title and the claim of interdisciplinarity one misses somewhat more references to some bioenergetic and microenergetic phenomena (e.g. photosynthesis), to the disputed relation of energetics and information theory; to the energetics of non--linear processes far from equilibrium and that of self--organizing, dissipative and ageing systems, etc.

The work consists of 10 chapters following a Preface and Table of Contents. At its end are: List of Symbols, three Appendices and a Subject Index. At the end of each chapter good exercises are found and problems that are to be solved, and here are the well selected references, though the selection is sometimes too onesided.

Chapter I (Fundamental Concepts) gives the thermodynamical definitions and postulates in encyclopedical key-words. Its sign convention is not the traditional one. While it is system--centric with regard to heat transfer it is antropocentric in considering work (i.e. the work produced by the machine is positive). The definitions of internal energy and exergy, too, are different from those of some other works. The main points of the postulates:

1/ In a stable thermodynamic equilibrium a system can not change spontaneously and can not produce work either. The

exergy depends only on the state of the system.

2/ The total energy of a system with respect to a reservoir of OK temperature is its maximal energy.

3/ The heat flowing into the system equals the sum of the energy change of the system and the work output.

Chapter II (Exergy) deals with the calculation of the maximum work to be gained from known parameters and with the problems of efficiency or economicalness. The relation of exergy to classical thermodynamic functions is discussed here and the more detailed description of this central concept is also given in this chapter. The exergy (Λ) is:

$$\Lambda = (E - E_o) - T_o(S - S_o) + p_o(V - V_o),$$

where E is the total energy, S the entropy, V the volume and the subscript o denotes the standard values in equilibrium with the reservoir.

For an isothermal and isobaric change $\Delta\Lambda = \Delta G$ i.e. the change of exergy is equal to the change of the Gibbs free energy. After the energy balance and exergy-accounting of different systems the author shows that in irreversible processes the rate of exergy destruction equals the product of the reservoir temperature and the rate of entropy creation. The relation is described by the Maxwell-Gouy-Stodola equation. Introducing the concept of thermergy the author proves that the efficiency of hot-water house-heating is five times higher than that of electric heating in the same conditions. Another example for economical optimization is the desalination of brackish water by electrodialysis on ion-selective membranes.

Chapter III (Generalized Forces) describes the generalized driving forces, the conjugated flows and fluxes in the way usual in nonequilibrium thermodynamics. A novel feature is the application of exergy destruction proportional to entropy creation ($-\dot{\Lambda} = T_o\dot{S}$).

Chapter IV (Isothermal Flow Coupling) already presents the applications of the theory in more practical cases: it shows the simultaneous effect of noncentration, electric potential and pressure gradients on the transport in ion--exchange membranes, i.e. the electrokinetic cross-effects.

The friction model characterizes the interactions of the components thus, to some extent, the mechanism as well.

Chapter V (Conductance Coefficients and Reciprocity Relations), Chapter VI (General Energetics of Chemical Reactions) and Chapter VII (Interdiffusion of Gases in Porous Media) apply to further special cases the theory based on exergy.

Chapter VIII (Molecular Filtration Throught Membranes) is focused on the discussion of the reverse osmotic, desalination transport processes. This chapter is an outstanding example for the unity of theory and experience, and at the same time shows the life-condition creating benefits of basic research.

Chapter IV (Coupled Heat and Mass Flow) and Chapter X (Thermoelectric Phenomena) discuss briefly thermal effects debated by biophysicists since long ago: thermal diffusion, the Dufour effect, thermoosmosis, etc. Their selection of literature is biased and incomplete concerning the model in general, and it is even more so for the biological aspects.

Summarizing it can be stated that the work is a short, compact and good review of the energetics of linear transport processes based on exergy mainly for engineering-minded readers.

F. Vetõ

Printed in Hungary
Akadémiai Kiadó és Nyomda, Budapest

Biological Chemistry Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

This Journal was founded in 1877 as *Zeitschrift für Physiologische Chemie* by F. Hoppe-Seyler and was continued after his death under the editorship of A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* [Volume 21 (1895) - Volume 365 (1984)].

Subscription price: 1986, Volume 367 (12 issues): DM 880,–

Volume 367 | February 1986 | Number 2

Walter de Gruyter · Berlin · New York

Biological Chemistry Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

This Journal was founded in 1877 as *Zeitschrift für Physiologische Chemie* by F. Hoppe-Seyler and was continued after his death under the editorship of A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* [Volume 21 (1895) – Volume 365 (1984)].

Subscription price: 1986, Volume 367 (12 issues): DM 880,–

Volume 367 | April 1986 | Number 4

Walter de Gruyter · Berlin · New York

A desirable plan for the organization of a paper is the following: Summary, Introduction, Materials and methods, Results, Discussion, References.

A clear and informative title is very important. Provide a short title (not to exceed 50 characters and spaces) to be used as a running head. Every paper must begin with a **Summary** (up to 200 words) presenting the important and pertinent facts described in the paper.

The **Introduction** should state the purpose of the investigation, but should not include an extensive review of the literature. The description of the **Materials and methods** should be brief, but adequate for repetition of the work. Refer to previously published procedures employed in the work. It is strongly recommended that author(s) should draw attention to any particular chemical or biological *hazards* that may occur in carrying out experiments described in the paper. Relevant safety precautions should be suggested. The **Results** may be presented in tables or figures. The **Discussion** should be concise and deal with the interpretation of the results. In some cases combining **Results and discussion** in a single section may give a clearer presentation.

References to the literature in the text should be by numbers in parentheses. In the reference list the items should be arranged in order of these serial numbers. For citation note the following examples:

1. Drust, D. S. and Martin, T. F. J. (1985) Biochem. Biophys. Res. Commun. 128, 531–537
2. Hoyer, S. (1980) in Biochemistry of Dementia (Roberts, P. J. ed.) pp. 252–257, J. Wiley and Sons, New York

Abbreviations and symbols defined in the IUPAC-IUB Document No. 1 (Arch. Biochem, Biophys. *115*, 1–12, 1966) may be used without definition, but others are to be avoided. When necessary, *abbreviations must be defined in a footnote and typed single-spaced on a separate page*, rather than in the text. Abbreviations *must not* be used in the Summary. Enzyme names should not be abbreviated except when the substrate has an accepted abbreviation (e.g. ATPase). Use of Enzyme Commission (EC) code number is required when available. Styling of isotopes should follow the recommendations of the IUB Commission of Editors. The symbol of the isotope should be placed in brackets attached directly to the front of the name, e.g. [^{32}P] AMP. Isotope number should only be used as a (superior) prefix to the atomic symbol, not to an abbreviation.

Typing of manuscript

The accepted articles are reproduced directly from the submitted manuscript as camera ready copy, thus *no changes can be made in the manuscript after acceptance*. Therefore black silk or carbon typewriter ribbon should be used. For obvious reasons proofs are not required and cannot be supplied. Special care should be taken to ensure a sharp, clean impression of letters throughout the whole manuscript. It is strongly recommended to use an *electric typewriter*. Erasures or other corrections should be avoided. Spelling, word division and punctuation must be carefully controlled.

The title of the paper should be typed in capital letters near the top of the first page, with the name(s) an affiliation(s) of the author(s) just typed below. Manuscripts should be double-spaced; methods, references, abbreviations, footnotes and figure legends should be *single-spaced*. Typing area of the page must be as close as possible to 16 × 24 cm.

Each page should be numbered at the bottom in light blue pencil marks. On a separate sheet indicate a running title, not more than 6 key-words for subject indexing and the author to whom reprints are to be sent, including a telephone number if possible.

Illustrations and tables. Do not attempt to insert figures, figure legends or tables into the text. This will be done by the Publisher. Original drawings should be clearly labelled in black ink in a manner suitable for *direct reproduction*. Typewritten lettering is not acceptable. Figures will be reduced to fit within the area of the page. All letters, numbers and symbols should be drawn to be at least 2.5 mm high after reduction. Glossy photographs or drawings are acceptable provided they are sharp, clear prints with an even black density. Illustrations in colour cannot be accepted. Mathematical and chemical symbols that cannot be typed should be drawn carefully in black. Tables and illustrations should be submitted on separate sheets.

One hundred reprints will be supplied free of charge.

CONTENTS

Index: 26.030

FORMERLY ACTA BIOCHIMICA ET BIOPHYSICA ACADEMIAE SCIENTIARUM HUNGARICAE

Acta Biochimica et Biophysica Hungarica

VOLUME 21, NUMBER 3, 1986

Akadémiai Kiadó, Budapest
ABBPAP 21 (3) 159—306 (1986) HU ISSN 0001-5253

Acta Biochimica et Biophysica Hungarica

a Quarterly of the Hungarian Academy of Sciences

Editors

P. ELŐDI and J. TIGYI

Managing editors

P. GERGELY and A. NIEDETZKY

Acta Biochimica et Biophysica Hungarica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

H-1054 Budapest, Alkotmány u. 21

Subscription information

Orders should be addressed to

KULTURA Foreign Trading Company

H-1389 Budapest P.O.Box 149

or to its representatives abroad.

Acta Biochimica et Biophysica Hungarica is indexed in Current Contents

INSTRUCTION TO AUTHORS

Acta Biochimica et Biophysica Hungarica will primarily publish original and significant papers from Hungarian research institutes, universities and other laboratories. Original papers written in English in diverse fields of modern experimental biology will be considered for publication. Novel aspects of the information described in the paper should be clearly emphasized.

Manuscripts (one original and two copies) should be submitted to

Dr. P. Gergely, Department of Medical Chemistry, University Medical School, 4026 Debrecen, POB 7, Hungary *(Biochemistry)*

or

Dr. A. Nidetzky, Department of Biophysics, University Medical School, 7624 Pécs, POB 99, Hungary *(Biophysics)*.

To ensure rapid and accurate publication, the author(s) are invited to follow the instructions described below. Manuscripts which do not conform these rules will be returned.

Preparation of manuscript

Full-length papers should not exceed 10 typewritten pages on A4 size high quality paper. The complete manuscript including the space occupied by figures, tables, references, acknowledgement etc. should not exceed 15 pages.

Short communications of total length of 5 pages will also be accepted.

Acta Biochim. Biophys. Hung. 21(3), pp. 159-176 (1986)

PROTEIN PHOSPHORYLATION AND ASSOCIATIVE LEARNING IN HERMISSENDA*

J.T. Neary and D.L. Alkon

Section on Neural Systems, Laboratory of Biophysics, IRP
National Institute of Neurological and Communicative
Disorders and Stroke National Institutes of Health at the
Marine Biological Laboratory
Woods Hole, Massachusetts 02543 U.S.A.

(Received May 15, 1985)

SUMMARY

Phototaxis in the nudibranch mollusc Hermissenda crassicornis can be modified by a conditioning paradigm which consists of paired light and rotation. Changes in K^+ currents and protein phosphorylation have been demonstrated in photoreceptors and eyes of conditioned animals. A working hypothesis has been proposed in which stimulation of photoreceptors and hair cells during the presentation of paired light and rotation leads to synaptic and light-induced depolarization which gives rise to an increase in internal free Ca^{2+} in photoreceptors. The conditioning-induced increase in Ca^{2+} can in turn activate protein phosphorylation mechanisms which regulate K^+ channel activity. In experiments designed to test this hypothesis, we have found that (1) light plus depolarization increases internal Ca^{2+}, as measured by Arsenazo absorption, (2) Ca^{2+} increases the rate of inactivation of the same K^+ currents which are reduced following conditioning, (3) intracellular injection of Ca^{2+}/calmodulin-dependent and cAMP-dependent protein kinases leads to reductions in these K^+ currents, and (4) potent Ca^{2+}- and cAMP-dependent protein kinases are present in Hermissenda neural tissue. Further studies are needed (a) to investigate the nature of the proteins whose levels of phosphorylation are altered following training and the nature of the proteins that are affected by the intracellularly injected protein kinases and (b) to determine if the phosphoproteins affected by conditioning are related to the function of K^+ channels.

*Paper presented at the round table discussion on "Protein Phosphorylation and Memory Mechanisms" organized in the frame of the 5th Meeting of the European Society for Neurochemistry, Budapest, August 21-24, 1984.

Akadémiai Kiadó, Budapest

INTRODUCTION

Over the past few years, we and our colleagues have been investigating the question of how protein phosphorylation is related to learning and memory mechanisms in the nudibranch mollusc, Hermissenda crassicornis. Our experimental approach has been (1) to make observations on electrophysiological and biochemical processes in conditioned animals and then (2) to investigate mechanisms which regulate these processes by simulating the effects of conditioning. As described in the following sections, the results of our studies to date indicate that K^+ currents and phosphoproteins are modified by conditioning and suggest that one way in which protein phosphorylation can be involved in learning and memory mechanisms is by means of Ca^{2+}-mediated protein kinase regulation of K^+ channel activity.

1. Electrophysiological and biochemical correlates of associative learning in Hermissenda

Our laboratory is engaged in a behavioral, electrophysiological, and biochemical analysis of associative learning in the nudibranch mollusc, Hermissenda crassicornis (Alkon, 1980, 1983). A model of associative learning has been developed which results in a modification of the phototactic response, i.e., animals can be conditioned to take significantly longer times to locomote to a region of illumination. The increase in latency to move toward a light spot is dependent on the temporal pairing of two stimuli, light and rotation (Crow and Alkon, 1978). Conditioning with paired light and rotation results in a much larger latency than the presentation of random or unpaired light and rotation. Training consists of 50 or 100 trials of paired light and rotation per day for 3 to 4 days. The duration of each trial is 30 seconds with a variable intertrial interval of 1 to 3 minutes. Details of the automated training and testing procedure and apparatus have been documented previously (Crow and Alkon, 1978; Tyndale and Crow, 1979). A nonassociative component of the response to paired light and rotation is present after training (Alkon, 1974a), but the nonassociative response decreases rapidly during the first hour following training (Crow, 1983).

During stimulation with light and rotation, photoreceptors receive synaptic input from hair cells in the gravity-sensing organ, the statocyst (Alkon, 1973a, b; 1974b; 1975; Akaike and Alkon, 1980), and a number of electrophysiological correlates of the modified behavioral response have been found in the Type B photoreceptors (Crow and Alkon, 1980; West et al., 1982; Alkon et al., 1982a; Farley and Alkon, 1982; Forman et al., 1984). (The eye of Hermissenda consists of five photoreceptors - two Type A and three Type B cells; the Type A and B photoreceptors are distinguished by their electrophysiological properties.) The effects of conditioning on Type B photoreceptors include a tonic, light-evoked membrane depolarization, an increase in membrane depolarization in the tail of the generator potential in response to light, and increases in input resistance and spontaneous spike frequency in the dark following acquisition. When tested one to two days after termination of conditioning, the electrophysiological effects include an increase in input resistance, an increase in membrane depolarization in the tail of the generator potential in response to light, and a reduction in the early K^+ current (I_A). Additional studies indicate that other membrane currents also change with conditioning, as has recently been proposed (Alkon, 1982-83; Crow, 1983). It has now been shown that a Ca^{2+}-dependent K^+ current (I_C) is reduced following conditioning (Farley et al., 1984; Forman et al., 1984).

In order to extend these studies to the molecular level, we initiated a series of biochemical investigations using eyes from conditioned (paired) and control (random, unpaired and naive) animals. The first biochemical process chosen for study was protein phosphorylation. We found that several hours following acquisition (a time period in which the associative effects of paired light and rotation are expressed; Crow, 1983), there was a significant increase in ^{32}P incorporation in a phosphoprotein with an apparent molecular weight (Mr) of 20 000 (20K PP) in the eyes of animals whose phototactic behavior had been modified by pairing light and rotation (Fig. 1, Table 1; Neary et al., 1981). No significant difference in ^{32}P corpora-

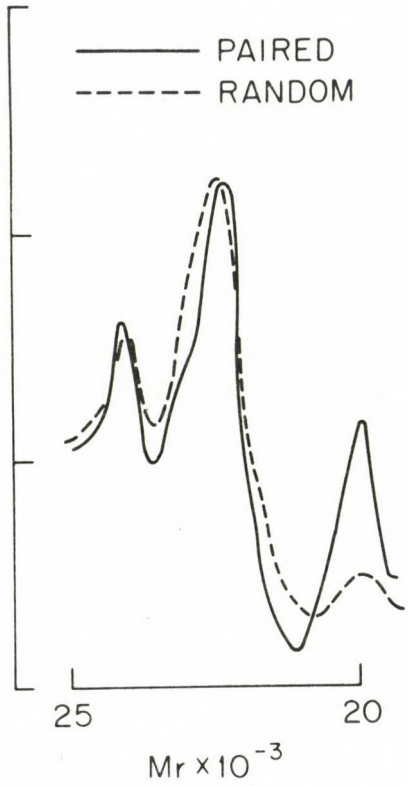


Fig. 1. Densitometric scans of the 25 000-20 000 Mr region of autoradiograms of eye samples obtained from animals receiving paired (solid line) or random (dashed line) light and rotation (from Neary et al., 1981).

tion in 20K PP was found in eyes from animals receiving random or unpaired stimuli. More recently, in preliminary studies with Drs. June Harrigan and Shigetaka Naito in our laboratory, we have employed two-dimensional gel electrophoresis and have found an increase in ^{32}P incorporation in a 55 000 Mr phosphoprotein, and possibly in a 22 000 Mr phosphoprotein, in eyes of animals conditioned to paired light and rotation and analyzed 24 hours after the termination of training (Alkon et al., 1984). As the eye of Hermissenda consists of only five photoreceptors, a lens, and pigment and epithelial cells, a change in protein phosphorylation correlated with associative learning has been localized to a few cells within a nervous system.

Table 1. Effect of paired, random, and unpaired stimulation on ^{32}P incorporation in specific phosphoprotein in Hermissenda eyes[a].

PHOSPHOPROTEIN BAND (Mr)	RATIO OF EXPERIMENTAL/UNTREATED (MEAN±SEM) Paired (N=16)	Random (N=12)	Unpaired (N=5)
72 000	0.83±0.08	1.05±0.23	1.30±0.38
55 000	1.02±0.04	1.04±0.09	0.88±0.07
44 000	0.88±0.12	1.35±0.10	1.04±0.44
42 000	1.27±0.14	1.18±0.08	1.08±0.13
38 000	1.11±0.07	0.87±0.10	1.23±0.14
34 000	1.55±0.21	1.22±0.20	1.56±0.32
31 000	1.12±0.14	1.23±0.18	0.91±0.16
29 000	0.89±0.11	1.24±0.17	1.15±0.41
24 000	1.12±0.17	0.90±0.15	1.10±0.18
22 000	1.04±0.10	1.16±0.14	0.76±0.07
20 000	[b]2.16±0.28	1.13±0.10	1.18±0.14

[a] For experimental details, see Neary et al., 1981.

[b] $F_{2.30} = 6.49$; $P<0.01$.

Thus, a number of observations from Hermissenda whose phototactic response has been modified by the temporal pairing of stimuli are consistent with changes in K^+ currents and protein phosphorylation. These studies provide the basis for detailed investigations of the mechanisms involved in associative learning in Hermissenda and how these mechanisms are regulated.

2. Studies of mechanisms underlying associative learning in Hermissenda

What biophysical and biochemical mechanisms give rise to the observed changes in the photoreceptors and eyes of conditioned Hermissenda? As described in the previous section, our studies to date indicate an important role for K^+ currents and protein phosphorylation. In addition, investigations in several laboratories, including our own, have shown that ionic currents can be modified by protein phpsphorylation (Castellucci et al., 1980; Kaczmarek et al., 1980; Levitan and Adams, 1981; DePeyer et al., 1982; Adams and Levitan, 1982; Strumwasser et al., 1982; Castellucci et al., 1982; Osterrieder et al., 1982; Alkon et al. 1983a; Shimoda et al., 1983; Camardo et al., 1983; Acosta-Urquidi et al., 1984a). Hence, some of our recent experiments have focused on the regulation of K^+ channel activity by protein phosphorylation.

A key feature of our working hypothesis of mechanisms underlying conditioning-induced biophysical and biochemical changes involves an increase in intracellular Ca^{2+} in Type B photoreceptors (see Fig. 2). During paired stimulation with light and rotation, hair cells in the gravity-sensing organ, the statocyst, cause brief inhibitory followed by prolonged excitatory synaptic input in Type B photoreceptors. This synaptic input, when coupled with the light-induced stimulation of the photoreceptors during the repetition of paired conditioning, leads to a persistent increase in membrane polarization (Crow and Alkon, 1980; Alkon, 1980b). (When stimulation of hair cells and photoreceptors are uncoupled in time, no such persistent increase occurs.) The increase in membrane depolarization can then give rise to an increase in intracellular

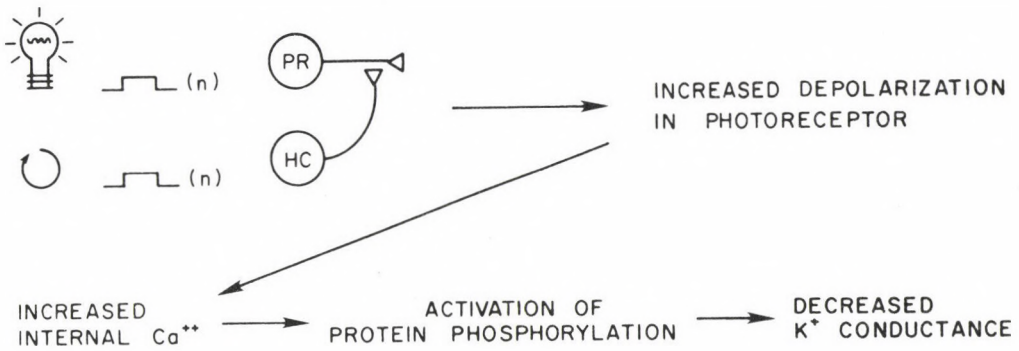


Fig. 2. Proposed sequence of events involved in conditioning-induced changes in K^+ currents

Ca^{2+}, as detected by an increase in Arsenazo dye signal, is enhanced in axotomized Type B photoreceptors by pairing light with depolarization (Connor and Alkon, 1984). (The depolarizing current delivered to the axotomized photoreceptor simulated the excitatory input of the hair cells during rotation.)

The paired stimuli-induced rise in intracellular Ca^{2+} can, in turn, affect the K^+ currents shown to change following modification of the phototactic response. Investigations of the properties of these currents have shown that Ca^{2+} increases the rate of inactivation of I_A (Alkon et al., 1982b). Secondly, studies on the Ca^{2+}-dependent K^+ current (I_C) suggest that the inactivation of this current is also increased by Ca^{2+} (Alkon and Sakakibara, 1984). Thus, it seems reasonable to suggest that the observed reductions of I_A and I_C following conditioning with paired light and rotation are brought about by a rise in intracellular Ca^{2+}.

One possible biochemical mechanism that could underlie a Ca^{2+}-induced inactivation of K^+ currents is that of Ca^{2+}/calmodulin-dependent protein phosphorylation. The Ca^{2+}-calmodulin complex could active protein phosphorylation/dephosphorylation directly by acting on Ca^{2+}-dependent protein kinases and phosphatases or indirectly by acting on Ca^{2+}-activated adenylate cyclase which in turn could active cAMP-dependent protein kinase. In order to test the effects of protein phosphorylation on K^+ currents, we have injected Ca^{2+}/calmodulin-dependent and

cAMP - dependent protein kinases into Type B photoreceptors and measured their effects on K^+ currents under voltage clamp conditions. As described below, the results of these studies show that intracellular injection of Ca^{2+}/calmodulin-dependent and cAMP-dependent protein kinase can mimic the biophysical effects of conditioning on Hermissenda photoreceptors.

Intracellular injection of a Ca^{2+}/calmodulin-dependent protein kinase, phosphorylase kinase from rabbit muscle (kindly supplied by Dr. E.G. Krebs and associates), leads to an increase in input resistance and to reductions in early and late K^+ currents (Acosta-Urquidi et al., 1984a). These effects are dependent on Ca^{2+}: (a) omission of Ca^{2+} from the bathing medium prevents the phosphorylase kinase mediated increase in input resistance, and (b) under voltage clamp, phosphorylase kinase reduces K^+ currents when a Ca^{2+} load is delivered to the cell by pairing light and depolarization (Fig. 3).

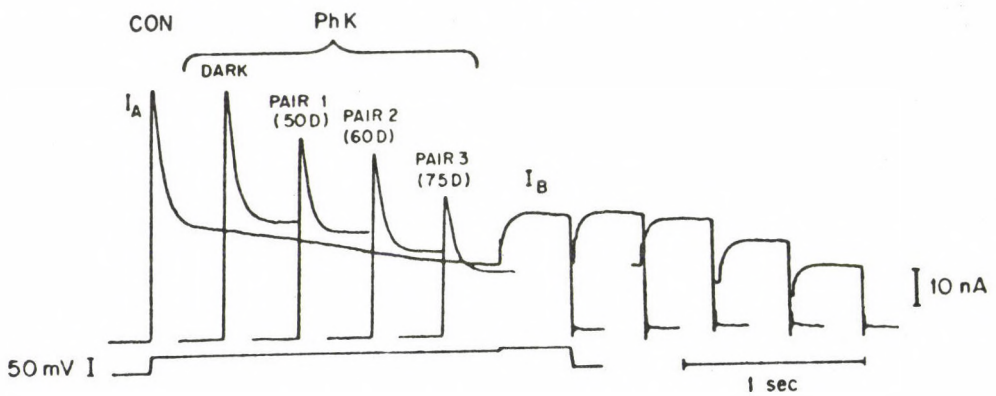


Fig. 3. Reduction of K^+ currents in Type B photoreceptors by intracellular injection of phosphorylase kinase (PhK), a type of Ca^{2+}/calmodulin-dependent protein kinase. CON: control, before injection of phosphorylase kinase. PAIR refers to pairings of light and depolarization (D, mV). Records of late K^+ currents (I_B) on right are from the same experiment as on left, and are displaced for purposes of comparison (from Acosta-Urquidi et al., 1984a).

Similar effects of phosphorylase kinase on K^+ currents have been observed in giant neurons, and the enzyme has also been shown to increase the Ca^{2+}-mediated inactivation of K^+ currents (Acosta-Urquidi et al., 1983). Furthermore, the calmodulin inhibitor calmidazolium (R24571) blocks the Ca^{2+} mediated inactivation of I_C (Alkon et al., 1984). Thus, the results of these experiments suggest a role for protein phosphorylation and calmodulin in mediating the effects of Ca^{2+} on K^+ currents.

A different type of protein kinase, cAMP-dependent protein kinase, also affects input resistance and K^+ currents. Injection of the catalytic subunit of bovine cAMP-dependent protein kinase (again kindly supplied by Dr. E.G. Krebs and associates) also reduced the early and late K^+ currents (Fig. 4) and increased input resistance (Alkon et al., 1983). In addition, similar Hermissenda neural proteins appear to be phosphorylated by the catalytic subunit of bovine heart cAMP-dependent protein kinase and by endogenous cAMP-dependent protein kinase (Neary et al., 1984).

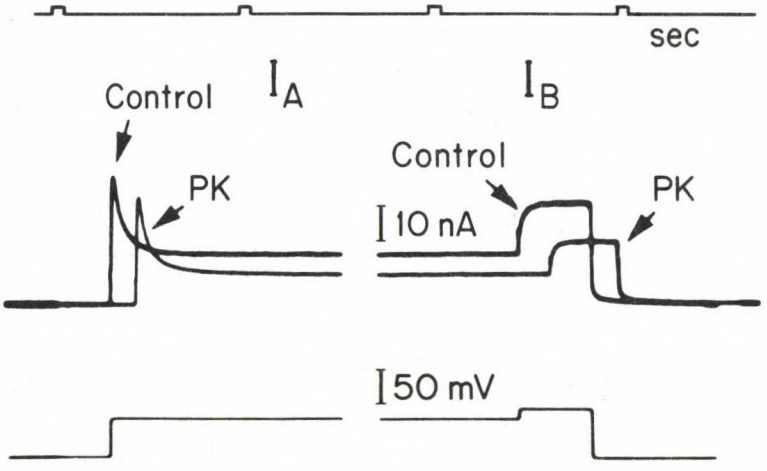


Fig. 4. Reduction of early (I_A) and late (I_B) K^+ currents in Type B photoreceptors by intracellular injection of the catalytic subunit (PK) of cAMP-dependent protein kinase (from Alkon et al., 1983).

A separate line of investigation also indicated a role for protein phosphorylation in the regulation of K^+ currents. This approach involves (a) the use of channel blockers which preferentially block specific types of ionic currents in molluscan neurons (for review, see Adams et al., 1980) and (b) the use of high external K^+ to inactivate voltage-dependent K^+ currents. Application of 4-aminopyridine (4-AP), which blocks I_A in molluscan neurons (Thompson, 1977, 1982; Byrne et al., 1979), to Hermissenda circumesophageal nervous system (CNS) that were previously labeled with ^{32}Pi leads to a marked reduction ($\geq$85%; Fig. 5) of ^{32}P incorporation in a 25 000 Mr phosphoprotein band (25K PP) in eyes and ganglia (Neary and Alkon, 1983). This effect occurred over a concentration range (1-10 mM) and time course (5-30 min) that are similar to those required to block I_A by 4-AP when the presence of the sheath surrounding the ganglia is taken into consideration. In addition, the effect of 4-AP is reversible; removal of 4-AP leads to an increase in ^{32}P incorporation in 25K PP and also in a 23 000 Mr phosphoprotein band (Fig. 6).

The effect of 4-AP on the level of ^{32}P in 25K PP does not appear to be the result of a 4-AP induced increase in impulse activity which can occur during 4-AP treatment (for review of effects of aminopyridines on synaptic transmission, see Thesleff, 1980). Neurons in the eye were isolated from the site of initiation of impulse activity and synaptic inputs by a lesion made in the optic tract between the eye and optic ganglia (Alkon, 1979). Eyes from the lesioned preparations still exhibited the 4-AP effect on 25K PP, thereby suggesting that the 4-AP effect on 25K PP cannot be explained by an indirect effect of 4-AP on impulse activity.

Since depolarization and increased internal Ca^{2+} are important features of our working hypothesis, we have also used high external K^+ to bring about sustained depolarization of Hermissenda neurons with an accompanying increase in internal Ca^{2+} and inactivation of the voltage-dependent K^+ currents. In one dimensional gel electrophoresis analysis, we have found that the 25K PP which is reduced by 4-AP is also reduced by

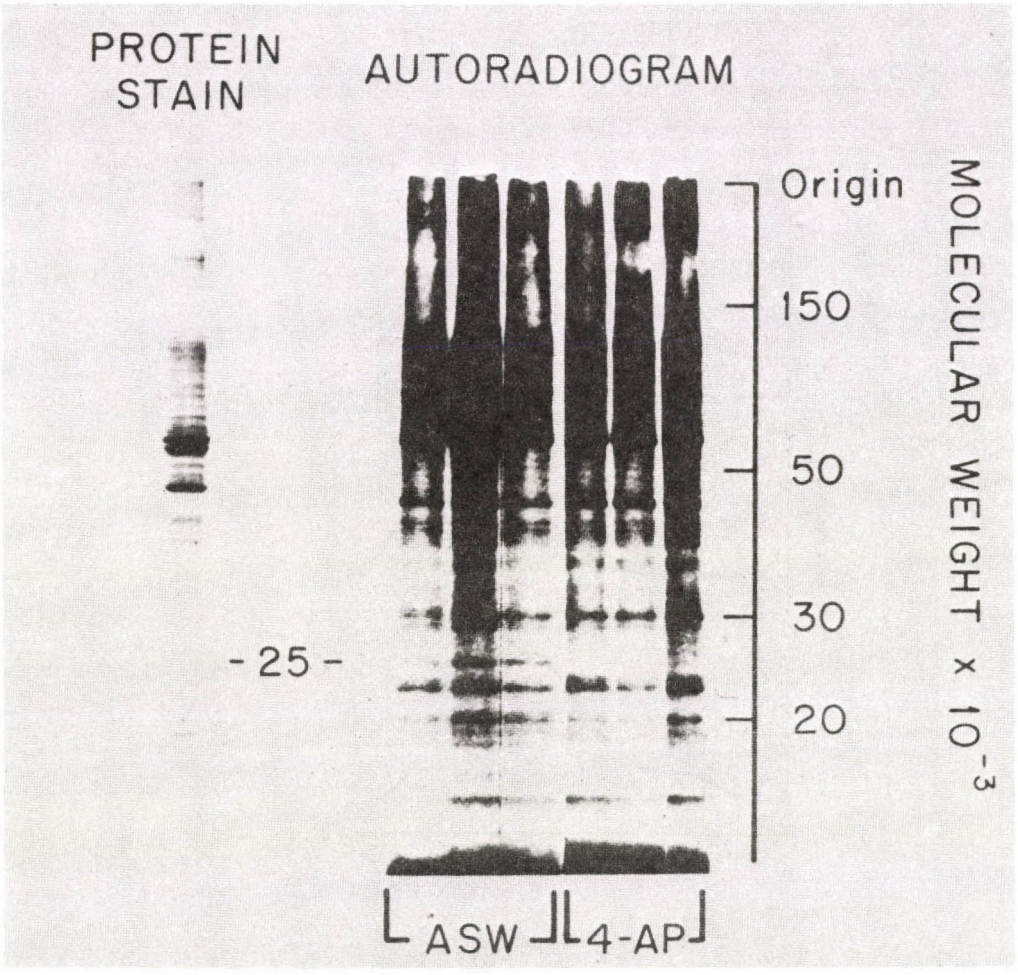


Fig. 5. Reduction in level of ^{32}P incorporation in 25K PP by 4-AP. Three different samples from controls (artificial sea water, ASW) and 4-AP experiments are shown to indicate sample to sample variations in the relative intensities of the phosphoprotein bands (from Neary and Alkon, 1983). Note decrease in 25K PP in 4-AP lanes.

sustained depolarization with 100 mM K^+ (Neary and Alkon, 1983). Two-dimensional gel electrophoresis analysis, conducted in collaboration with Dr. Shigetaka Naito of our laboratory, also reveals a dramatic increase in a 22K PP in response to sustained depolarization with high K^+. As mentioned previously, preliminary experiments suggest an increase in ^{32}P incorporation in this phosphoprotein in the eyes of Hermissenda con-

ditioned with paired light and rotation; thus, treatment with high K^+ may provide a model system to investigate some of the effects of conditioning.

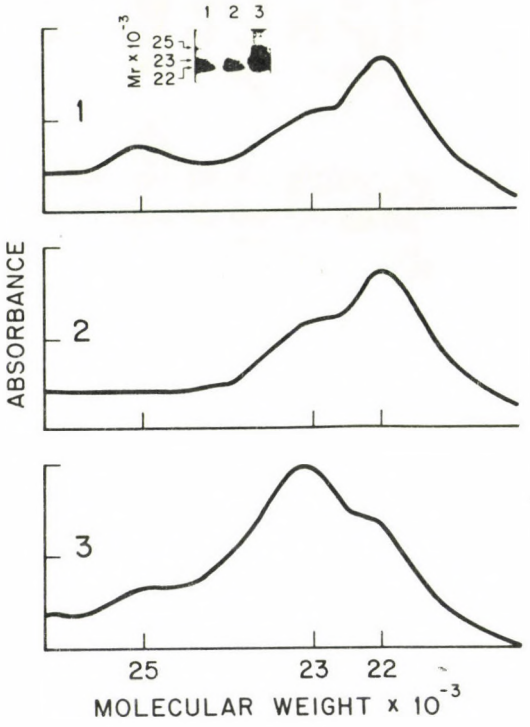


Fig. 6. Reversibility of 4-AP effect on 25K PP. Autoradiograms (inset) and densitometric scans are from experiments in which ^{32}P-labeled CNS were incubated (1) in ASW alone, (2) in 10 mM 4-AP for 30 min or (3) in ASW (30 min) following a 30 min incubation in 10 mM 4-AP (from Neary and Alkon, 1983).

Because results of our studies to date indicate a role for protein phosphorylation in regulating K^+ channel activity and in associative learning in Hermissenda, we have begun a series of experiments to characterize the protein kinases and their substrate proteins in Hermissenda neural tissue.

3. Studies of endogenous Ca^{2+}- and cAMP-dependent protein kinases and substrate proteins in Hermissenda neural tissue

In order to investigate the nature of (a) the proteins whose level of phosphorylation is altered following acquisition and retention of the phototactic response, (b) the proteins that are phosphorylated by the injected kinases and (c) the endogenous protein kinase and phosphatase activities that may regulate K^+ channel function in situ, we have begun to characterize the Ca^{2+}- and cAMP-dependent protein kinases and their substrate proteins in Hermissenda neural tissue (Neary et al., 1984).

Both Ca^{2+} and cAMP can stimulate the phosphorylation of many proteins in the 15 000 to 200 000 Mr range in CNS homogenates. The predominant Ca^{2+}- and cAMP-stimulated bands are 53 000 and 55 000 Mr, respectively. The predominant cAMP-stimulated band co-migrates on one-dimensional gels with a Ca^{2+}-stimulated phosphoprotein band. Many less-heavily phosphorylated proteins are also stimulated by Ca^{2+} and cAMP. As in the case of the 55 000 Mr protein, some of these proteins appear to be substrates for both Ca^{2+}- and cAMP-dependent protein kinases. Phosphorylation of a protein by both Ca^{2+}- and cAMP-dependent protein kinases has been reported in many vertebrate and invertebrate preparations (e.g., Huttner and Greengard, 1979; Lepeuch et al., 1979; Sieghart et al., 1979; Soderling et al., 1979; Walsh et al., 1979; Kennedy and Greengard, 1981; DeRiemer et al., 1982; Novak-Hofer and Levitan, 1983) and may serve as a convergence point for the Ca^{2+} and cAMP intracellular communication systems (see reviews by Rasmussen, 1981, and Cohen, 1982). Phosphorylation of the same proteins by Ca^{2+}- and cAMP-dependent protein kinases is consistent with our studies on the effects of injected kinases in photoreceptors in which both Ca^{2+}/calmodulin- and cAMP-dependent protein kinases affected the early and late K^+ currents. Further studies are needed to determine if the proteins that are common substrates for the endogenous Ca^{2+}-

and cAMP-dependent protein kinases are the same proteins that are phosphorylated by the injected kinases *in situ*.

Preliminary studies on the effects of free Ca^{2+} concentrations on protein phosphorylation/dephosphorylation in *Hermissenda* CNS indicate that the Ca^{2+}-dependent protein kinase responds to sub-micromolar free Ca^{2+} concentrations (Neary et al., 1984), i.e., levels of free Ca^{2+} that might be expected to occur upon stimulation. Increased ^{32}P incorporation in the major Ca^{2+} stimulated band (53 000 Mr) can be detected at $\leq$0.5 µM; incorporation in other bands occurs in the 1 to 20 µM range. At higher free Ca^{2+} concentrations (200-500 µM), ^{32}P incorporation is decreased in several bands. Ca^{2+}-activated proteolysis has not been detected on Coomassie-stained gels, although silver-staining of proteins is needed to determine if some minor proteins are degraded at the higher Ca^{2+} concentrations. Time-course studies indicate that Ca^{2+}-stimulated phosphorylation in several bands peaks at 1 to 2 minutes and declines thereafter, whereas in other bands ^{32}P incorporation remains relatively constant from 1 to 10 minutes. These results suggest the presence of phosphatase activities, some of which may be Ca^{2+}-dependent, and it is tempting to speculate that the regulation of K^+ channel activity may be related to Ca^{2+}-stimulation of protein kinases and protein phosphatases.

In other preparations, two types of Ca^{2+}-stimulated protein kinases have been reported (e.g. see review by Krebs, 1983), a Ca^{2+}/calmodulin-dependent protein kinase and a Ca^{2+}-activated/phospholipid-dependent protein kinase (C kinase) (Nishizuka and Takai, 1981), and efforts to identify the types of Ca^{2+}-dependent protein kinases in *Hermissenda* are in progress. Studies with calmodulin inhibitors suggest that some of the *Hermissenda* neural proteins are phosphorylated by an endogenous Ca^{2+}/calmodulin-dependent protein kinase (Neary et al., 1984; Alkon et al., 1984). Addition of calmidazolium (R24571) or trifluoperazine blocks Ca^{2+}-stimulated phosphorylation of many proteins in CNS homogenates, and addition of exogenous calmodulin can block the action of the calmidazolium and trifluoperazine. These experiments are particularly inter-

esting in view of the effect of calmodulin inhibitors on I_C, namely, to prevent the Ca^{2+}-mediated inactivation of I_C (Alkon et al., 1984). To confirm the identification of a Ca^{2+}/calmodulin-dependent protein kinase in Hermissenda, calmodulin depletion and reconstitution studies are needed. C kinase has been reported in Aplysia (DeRiemer et al., 1983) and other molluscan ganglia (Kuo et al., 1980), and investigations of a possible role for C kinase in K^+ channel function are planned in Hermissenda.

Recent preliminary studies have provided clues to the identity of one of the phosphoproteins that may be affected by conditioning and by injection of protein kinase. In collaboration with Drs. Robert DeLorenzo and James Goldenring of Yale University Medical School and Juan Acosta-Urquidi in our laboratory, we have found that injection of a purified Ca^{2+}/calmodulin-dependent protein kinase from rat brain which phosphorylates tubulin and microtubule-associated proteins (Goldenring et al., 1983) can significantly affect I_C and other ionic currents in giant neurons and that this enzyme can effect the level of phosphorylation of several Hermissenda proteins in CNS homogenates (Acosta-Urquidi et al., 1984b). Furthermore, in collaboration with Drs. Shigetaka Naito and June Harrigan in our laboratory, we have found that the level of ^{32}P incorporation in a 55 000 Mr phosphoprotein (and possibly in a 22 000 Mr phosphoprotein) is increased in the eyes of Hermissenda during retention of the modified phototactic response. The 55 000 Mr phosphoprotein has a similar Mr to a protein that can be phosphorylated by Ca^{2+} and cAMP and whose Ca^{2+}-stimulated phosphorylation can be blocked by calmodulin inhibitors. Results of preliminary studies with two-dimensional gel electrophoresis suggest that the 55K PP has a Mr similar to that of rat tubulin but is slightly more basic than rat tubulin. The small difference in isoelectric points may be due to a species difference. Further studies employing immunoprecipitation with tubulin antibody and peptide mapping techniques are needed to determine if the 55K PP is tubulin. A change in tubulin levels has been reported in chicks trained with a

passive avoidance paradigm (Mileusnic et al., 1980). The mechanism of tubulin and phosphorylated tubulin action in learning paradigms has not been elucidated; one possibility may involve the anchoring of cytoskeletal proteins to the plasma membrane for the alteration or augmentation of intracellular information flow.

Acknowledgements

We are grateful to our colleagues Juan Acosta-Urquidi, Terry Crow, Robert DeLorenzo, Susan DeRiemer, Hal Geiner, James Goldenring, June Harrigan, Len Kaczmare, Paul Kandel, Alan Kuzirian, Greg Kuzma, Shigetaka Naito, Jim Olds, Howard Rasmussen, Manabu Sakakibara, and Leslie Tengelsen, for their contributions to the work described here. We also thank Jeanne Kuzirian for secretarial support.

REFERENCES

Acosta-Urquidi, J., Alkon, D.L., Connor, J.A., Neary, J.T. (1983) Soc. Neurosci. Abstr. 9, 501

Acosta-Urquidi, J., Alkon, D.L., Neary, J.T. (1984a) Science 1254-1257

Acosta-Urquidi, J., Neary, J.T., Goldenring, J.R., Alkon, D.L., DeLorenzo, R.J. (1984b) Soc. Neurosci. Abstr. (in press)

Adams, D.J., Smith, S.J., Thompson, S.H. (1980) Ann. Rev. Neurosci. 3, 141-167

Adams, W.B., Levitan, I.B. (1982) Proc. Natl. Acad. Sci. USA 79, 3877-3880

Akaike, T, Alkon, D.L. (1980) J. Neurophysiol. 44, 501-513

Alkon, D.L. (1973a) J. Gen. Physiol. 61, 444-461

Alkon, D.L. (1973b) J. Gen. Physiol. 62, 185-202

Alkon, D.L. (1974a) J. Gen. Physiol. 64, 70-84

Alkon, D.L. (1974b) Fed. Proc. 33, 1083-1090

Alkon, D.L. (1985) J. Gen. Physiol. 65, 385-397

Alkon, D.L. (1979) Science 205, 810-816

Alkon, D.L. (1980a) Biol. Bull. 159, 505-560

Alkon, D.L. (1980b) Science 210, 1375-1376

Alkon, D.L. (1982-1983) J. Physiol. (Paris) 78, 700-706

Alkon, D.L. (1983) Sci. Amer. 249, 70-84

Alkon, D.L., Sakakibara, M. (1984) Soc. Neurosci. Abstr. (in press)

Alkon, D.L., Lederhendler, I., Shoukimas, J.J. (1982a) Science 215, 693-695

Alkon, D.L., Shoukimas, J.J., Heldman, E. (1982b) Biophys. J. 40, 245-250

Alkon, D.L., Acosta-Urquidi, J., Olds, J., Kuzma, G, Neary, J.T. (1983) Science 219, 303-306

Alkon, D.L., Naito, S., Neary, J., Harrigan, J., Sakakibara, M., Weiner, A., Heldman, E. (1984) Biol. Bull. Abstr. (in press)

Byrne, J.H., Shapiro, E., Dieringer, N., Koester, J. (1979) J. Neurophysiol. 42, 1233-1250

Camardo, J.S., Shuster, M., Siegelbaum, S.A., Eppler, C.M., Kandel, E.R. (1983) Soc. Neurosci. Abstr. 9, 22

Castellucci, V.F., Kandel, E.R., Schwartz, J.H., Wilson, F.D., Nairn, A.C., Greengard, P. (1980) Proc. Natl. Acad. Sci. USA, 77, 7492-7496

Castellucci, V.F., Nairn, A., Greengard, P., Schwartz, J.H., Kandel, E.R. (1982) J. Neurosci. 2, 1673-1681

Cohen, P. (1982) Nature 296, 613-620

Connor, J., Alkon, D.L. (1984) J. Neurophysiol. 51, 745-752

Crow, T. (1983) J. Neurosci. 3, 2621-2628

Crow, T.J., Alkon, D.L. (1978) Science 201, 1239-1241

Crow, T.J., Alkon, D.L. (1980) Science 209, 412-414

DePeyer, J.E., Cachelin, A.B., Levitan, I.B., Reuter, H. (1982) Proc. Natl. Acad. Sci. USA 79, 4207-4211

DeRiemer, S.A., Kaczmarek, L.K., Greengard, P. (1982) Soc. Neurosci. Abstr. 8, 565

DeRiemer, S.A., Kaczmarek, L.K., Albert, K.A., Greengard, P. (1983) Soc. Neurosci. Abstr. 9, 77

Farley, J., Alkon, D.L. (1982) J. Neurophysiol. 48, 785-807

Farley, J., Sakakibara, M., Alkon, D.L. (1984) Soc. Neurosci. Abstr. (in press)

Forman, R., Alkon, D.L., Sakakibara, M., Harrigan, J., Lederhendler, I., Farley, J. (1984) Soc. Neurosci. Abstr. (in press)

Goldenring, J.R., Gonzalez, B., McGuire, Jr., J.S., DeLorenzo, R.J. (1983) J. Biol. Chem. 258, 12632-12640

Huttner, W.B., Greengard, P. (1979) Proc. Natl. Acad. Sci. USA 76, 5402-5406

Kaczmarek, L.K., Jennings, K.R., Strumwasser, F., Narin, A.C., Walter, U., Wilson, F.D., Greengard, P. (1980) Proc. Natl. Acad. Sci. USA 77, 7487-7491

Kennedy, M.B., Greengard, P. (1981) Proc. Natl. Acad. Sci. USA 78, 1293-1297

Krebs, E.G. (1983) Phil. Trans. R. Soc. Lond. B302, 3-11

Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, L., Brackett, N.L., Katoh, N., Shoji, M., Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039-7043

LePeuch, C.J., Haiech, J., Demaille, J.G. (1979) Biochemistry 18, 5150-5157

Levitan, I.B., Adams, W.B. (1981) Adv. Cyclic Nucleotide Res. 14, 647-653

Mileusnic, R. Rose, S.P.R., Tillson, P. (1980) J. Neurochem. 34, 1007-1015

Neary, J.T., Alkon, D.L. (1983) J. Biol. Chem. 258, 8979-8983

Neary, J.T., Crow, T., Alkon, D.L. (1981) Nature 293, 658-660

Neary, J.T., DeRiemer, S.A., Kaczmarek,L.K., Alkon, D.L. (1984) Soc. Neurosci. Abstr. (in press)

Nishizuka, Y., Takai, Y. (1981) Cold Spring Harbor Conf. Cell Proliferation 8, 237-249

Novak-Hofer, I., Levitan, I.B. (1983) J. Neurosci. 3, 473-481

Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V., Hofmann, F. (1982) Nature 298, 576-578

Rasmussen, H. (1981) in Calcium and cAMP as synarchic messengers, Wiley, New York

Shimoda, Y., Miller, W.H., Lewis, R.M., Nairn, A.C., Greengard, P. (1983) Soc. Neurosci. Abstr. 9, 77

Sieghart, W., Forn, J., Greengard, P. (1979) Proc. Natl. Acad. Sci. USA 76, 2475-2479

Soderling, T.R., Srivastava, A.K., Bass, M.A., Khatra, B.S. (1979) Proc. Natl. Acad. Sci. USA 76, 2536-2540

Strumwasser, F., Kaczmarek, L.K., Jennings, K.R. (1982) Fed. Proc. 41, 2933-2939

Thesleff, S. (1980) Neuroscience 5, 1413-1419

Thompson, S.H. (1977) J. Physiol. (London) 265, 465-488

Thompson, S.H. (1982) J. Gen. Physiol. 80, 1-18

Tyndale, C.L., Crow, T. (1979) IEEE Trans. Biomed. Eng. 26, 649-655

Walsh, K.X., Millikin, D.M., Schlender, K.K., Reimann, E.M. (1979) J. Biol. Chem. 254, 6611-6616

West, A., Barnes, E.S., Alkon, D.L. (1982) J. Neurophysiol. 48, 1243-1255

Acta Biochim. Biophys. Hung. 21(3), pp. 177-192 (1986)

DEFECTIVE cAMP METABOLISM AND DEFECTIVE MEMORY IN DROSOPHILA*

Y. Dudai, J. Buxbaum, G. Corfas, S. Orgad,
D. Segal, B. Sher, A. Uzzan, S. Zvi

Department of Neurobiology, The Weizmann Institute of Science,
Rehovot 76100, Israel

(Received May 15, 1985)

1. GENETIC DISSECTION OF LEARNING AND MEMORY

Learning and memory are expected to be based on alterations in the nervous system of the organism that learns and remembers. Current theories suggest that these alterations affect the flow of information in the neuronal networks that execute the modified responses, more specifically, in synaptic connections within the network. Since the synapses involved in a given learning process may comprise only a very minute fraction of the total synapses in the nervous system, the identification and characterization of the cellular, subcellular and molecular alterations that take place in the learning process may become an extremely difficult task. Direct investigation of the activities of nerve cells and their processes is feasible only in a few cases, e.g., when the network is a relatively simple subsystem of a complex nervous system, or when animals with relatively simple nervous systems and large neurons are encountered (e.g., certain molluscs). In our studies of the molecular mechanisms of learning and memory, we are pursuing a different strategy, which partially circumvents the necessity

*Paper presented at the round table discussion on "Protein Phosphorylation and Memory Mechanisms" organized in the frame of the 5th Meeting of the European Society for Neurochemistry, Budapest, August 21-24, 1984.

Akadémiai Kiadó, Budapest

to pinpoint a priori the anatomical locus of memory in order to identify the molecular apparatus involved. This indirect strategy is the genetic dissection of learning and memory.

The rationale for the genetic dissection approach to memory is simple (Aceves-Pina et al., 1983; Dudai, 1979; Dudai et al., 1976; Quinn et al., 1974): genes are assumed to code for macromolecules that comprise the building blocks of biological memory systems. By altering each of the appropriate genes separately, one should be able to produce specific lesions in the components of the learning apparatus, thus disrupting the ability to learn and remember. Genetic, biochemical and electrophysiological comparisons of mutant and normal organisms, which differ in one gene only, may reveal the nature of the gene product, and hence of a molecular component required for normal learning. There is, of course, an additional assumption which underlies the approach, namely, that gene products exist whose function is important for learning and memory much more than for other behavioral (or developmental) processes.

The experimental approach is thus as follows: One induces mutations in the organism, e.g., by a chemical mutagen, and tests the effect of the yet unidentified mutational events on the performance in a learning and memory task. If a mutation leads to inability to learn and/or remember, and the abnormal behavior cannot be ascribed to trivial motor or sensory defects (e.g., inability to detect sensory cues or to execute motor acts which are required for the appropriate response), then the genetic, behavioral and molecular bases of the defect are further investigated. The experimental organism suitable for the genetic approach should be capable of learning, and readily amenable to genetic analysis. The fruit fly, Drosophila melanogaster fulfills these requirements.

The learning paradigm which has been used to date as a screening assay for learning mutants in Drosophila is an olfactory conditioning task, in which the flies are required to avoid an odorant coupled to an electric shock. The paradigm, in various versions, has been described in detail (Dudai, 1977; Dudai, 1979; Quinn et al., 1974). In brief, populations of

flies (30-50 each) are driven by phototaxis into an electrified grid covered with a solution of a certain odorant (e.g., 3-octanol or amylacetate), or into a non-electrified grid covered with a control odorant. The flies are thus conditioned to associate an olfactory cue with an aversive stimulus. During testing, the flies are tested for their avoidance of the shock-associated odorant (this time on a new grid and with no shock), and of the control odorant. The fraction of flies avoiding each odorant during testing is determined. To control for odor bias, the experiment is repeated with a second population of flies, but with the roles of shock-associated and control odorants being reversed. A learning index, λ, which is a measure of the specific odorant-avoidance acquired during training, is defined as the fraction of the population avoiding the shock-associated odorant during testing, minus the fraction avoiding the control odorant, averaged for the two reciprocal halves of an experiment. A learning index of $\lambda=0$ indicates no learning, and $\lambda=1.0$ indicates perfect conditioning. For the wild type Canton-S (C-S), which was used to date to generate conditioning mutants, $\lambda=0.4$ when tested within a few min after the end of training, and the half-life of memory is about 1 hr (both values are for a routine training procedure in which the flies are exposed 3 times to the shock, for 30 sec each time, with 30 to 90 sec interval between each training session; Dudai, 1977; Dudai et al., 1976; Quinn et al., 1974).

2. PHARMACOLOGY OF LEARNING IN DROSOPHILA

The effect of drugs on the performance of an organism in a learning paradigm can sometimes yield interesting hints as to the properties of the behavioral modification and the metabolic processes involved. We have studied, during the past few years, the effect of various pharmacological treatments on the leraning performance of Drosophila. These experiments could be divided into two types: Experiments in which the effect of the treatment on immediate memory (0.5-5.0 min after training) was monitored, and experiments in which the effect on delayed memory (e.g., 1 hr after training) was tested.

Many of the drugs tested, whidh are known to affect enzymes receptors and uptake systems in the nervous system of invertebrates and vertebrates (e.g., Dudai and Jan, 1975; Dudai et al., 1980), do affect, under the appropriate conditions, the learning behavior of Drosophila. However, in most of the cases, the drug affects many behaviors, e.g. locomotion, phototaxis, and therefore specific effect on learning cannot be established. However, exceptions do exist. Several drugs, at concentrations which start to severely affect the general behavior of the flies, do not significantly affect learning. Among them are neostigmine, a potent inhibitor of acetylcholinesterase, and picrotoxin, a GABA antogonist (Table 1). In contrast, drugs that under the conditions tested had no marked effects on general behavior, did decrease markedly the memory of the flies, when tested immediately after training; these drugs were caffeine (Dudai and Jan, 1975; Table 1), theophyline and IBMX, all inhibitors of cAMP phosphodiesterase in Drosophila (the same ligands display potent affinity for adenosine receptors in vertebtates, but as yet we have found no evidence for such receptors in Drosophila).

Table 1 also summarizes the effect of some treatments on delayed memory. A protein synthesis inhibitor, under conditions that block ≅ 90% of protein synthesis in Drosophila, had no effect on 1 hr memory (Dudai, 1977). On the other hand, cold or N_2 anaesthesia, if induced within ca. 20 min after training (but not afterwards), blocks 1 hr memory (Dudai, 1977; Quinn and Dudai, 1976).

The information obtained from pharmacological experiments is indeed a priori limited, since the drugs are not very specific, their in vivo targets are difficult to identify, and they tend to affect many metabolic processes and behaviors simultaneously. Nevertheless, the results on phosphodiesterase inhibitors seem to suggest that cAMP metabolism may play a role in Drosophila immediate memory; other results suggest that cholinergic and GABAergic transmission do not play a major role in immediate memory and that protein synthesis may not be important for memory up to a few hours after training. The results

obtained with cold and N_2 treatment suggest that memory in the olfactory paradigm is composed of at least two phases, the first of which is sensitive to anaesthesia, the latter is not.

Table 1. Effect of various pharmacological treatments on learning and memory in Drosophila

TREATMENT	MEMORY % of control
Neostigmine[1]	111
Picrotoxin[1]	93
Caffeine[1]	28*
Cycloheximide[2]	117
Cold anaesthesia[2]	20*
N_2 anaesthesia[2]	10*

Flies were fed on a 2% sucrose solution (immersed in Kimwipes Tissue Paper) containing the appropriate concentration of drug. The conditions used were: Neostigmine, 2 mg/ml for 20-24 hr; Picrotoxin, 0.05 µg/ml for 20-24 hr; caffeine, 1 mg/ml for 20-24 hr; cycloheximide, 5 mg/ml for 46 hr; cold and N_2 anaesthesia, 5 min each, within 15 min after training. Memory was tested 1.5-2.0 min after training (1) or 1 hr after training (2).

*) $P<0.05$ for the difference from control.

3. LEARNING MUTANTS AND THEIR MEMORY

Behavioral and pharmacological experiments can be carried out in many organisms, but Drosophila has a huge advantage when genetics is considered. Indeed the use of Drosophila in learning research is due to the possibility of generating putative conditioning mutants. Several such mutations have been found in the past few years (Aceves-Pina et al., 1983; Dudai et al., 1976). We are currently concentrating on two of them, dunce (Dudai, 1979; Dudai et al., 1976) and rutabaga (Duerr et al., 1982).

Both mutations were isolated in a deliberate screen for EMS-induced, X-linked learning mutants (dnc was isolated in Caltech, rut in Quinn's group in Princeton). These mutations display very low learning indices in the olfactory learning paradigm by which they were screened, when tested 90-180 sec after training. By using modifications of the olfactory conditioning paradigm, which enable detection of very short-lived and labile memory, we were able to demonstrate that both dnc and rut can form an association between cue and reinforcement, but memory decays very rapidly (Fig. 1). The mutants can therefore be practically defined as memory mutants, or, more specifically, as short-term memory mutants. Furhermore, we have

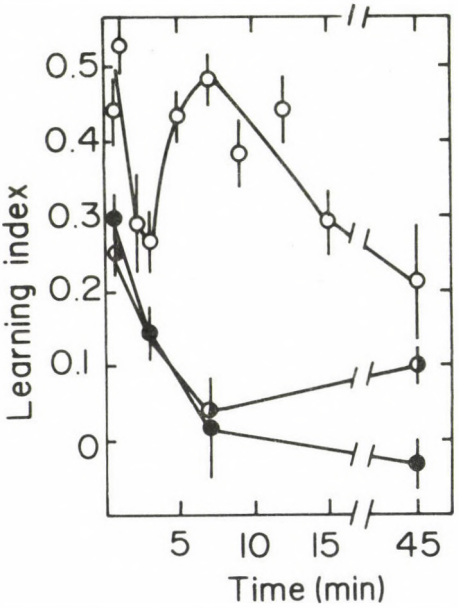


Fig. 1. dnc and rut are memory mutants. The figure depicts the memory decay curves of normal (o), dnc (●) and rut (◐) flies, following training in an olfactory conditioning negative reinforcement paradigm (data adapted from Dudai, 1983). It appears that the mutants can learn to avoid an odorant coupled to an electric shock (i.e., see memory immediately after training), but memory rapidly decays. Other behaviors of the mutants (e.g., phototaxis, odor discrimination) are apparently normal. For details of the conditioning paradigm and the definition of the learning index, see text.

been able to demonstrate, using "saving" experiments (in which the interaction of previously acquired memory with more recently acquired memory is tested), that dnc differs from rut: whereas in the latter mutant evidence was found for "mute" memory, which reveals itself only by interaction with newly acquired information in a conflict situation, no such evidence was detected in dnc (Dudai, 1983). Additional studies demonstrated that intensive training can improve the memory of rut when the memory is tested more than few minutes after training, but not if memory is tested less than a minute after training (Dudai et al., 1984). In conclusion, thus, it seems that both dnc and rut affect short-term memory processes, but they do it in a different way.

4. dnc HAS A DEFECTIVE cAMP-PHOSPHODIESTERASE

Preliminary genetic mapping of the dnc mutation was carried out upon its isolation (Dudai et al., 1976). Byers et al., 1981. extended the studies, located dnc within 3D3-4 region of the polythene X-chromosome, and showed that the gene very probably codes for an isozyme of cAMP-phosphodiesterase. Drosophila homogenates contain multiple forms of cAMP-phosphodiesterase. Only one of these forms, with high affinity for cAMP but low affinity for cGMP, is defective in dnc (Byers et al., 1981; Fig. 2.). The defect in dnc is detected in all body regions, and not only in the brain, although the affected form of the enzyme has high specific activity in the nervous system. We have recently succeeded in detecting cAMP-phosphodiesterase activity at the ultrastructural level in the nervous system of the fly, by the use of EM microhistochemical reactions; this may pave the way to subcellular localization of the dnc defect (Buxbaum and Dudai, unpublished).

5. rut HAS A DEFECTIVE ADENYLATE CYCLASE

In light of the results obtained by Byers et al. (1981) with dnc, we have decided to study additional facets of cAMP-metabolism in normal Drosophila and in learning and memory

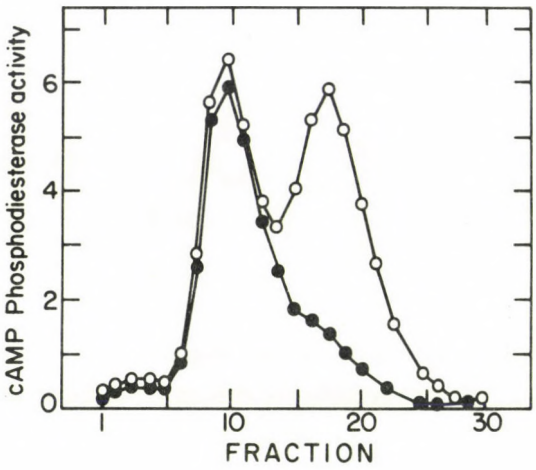


Fig. 2. The biochemical defect in dnc: cAMP-phosphodiesterase activity in homogenates of normal (o) and dnc (●) flies, subjected to centrifugation on a 5-20% sucrose gradient (with 60% sucrose as a cushion). One of the forms of the enzyme has a much reduced activity in the mutant, confirming the data of Byers et al. (1981). The top of the gradient is on the right-hand side.

mutants (Uzzan, 1981). A major component of the cAMP-cascade is adenylate cyclase (Schramm and Selinger, 1984). The activity of this enzyme was found to be defective in rut (Fig. 3; Dudai and Zvi, 1984; Dudai et al., 1983; Dudai et al., 1984; Livingstone et al., 1984). Again, as is the case with dnc, here too not the entire enzymatic activity was affected, but only what appeared to be a subpopulation, or a functional state, of the enzyme. Adenylate cyclase in homogenates of Drosophila is heterogeneous with respect to its affinity toward MgATP and its subcellular distribution. The majority of the enzyme is particulate. rut lacks up to 35% of the total particulate activity (the soluble activity is not affected). The defect is intimately associated with the catalytic subunit of the enzyme, and is manifested in a lower V_{max}, an altered K_m for MgATP, and altered responsiveness to forskolin, Mg^{2+} and Ca^{2+}. Of special interest (see below) is the defect in the Ca^{2+}-responsiveness. Calcium ions have a dual effect on adenylate cyclase system. In all systems described to date, high Ca^{2+} (>1 μM) inhibits the

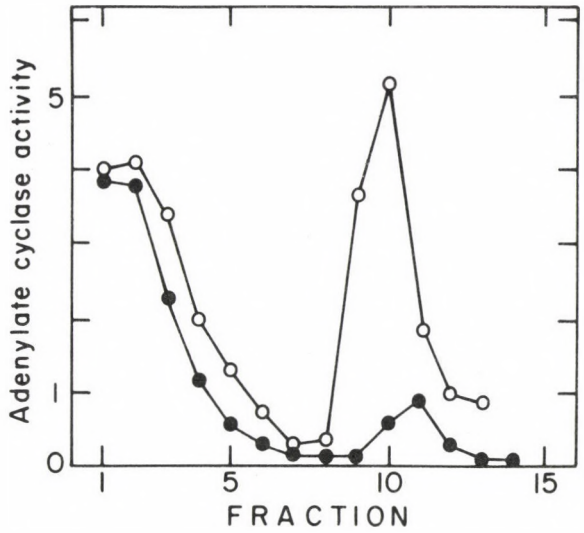


Fig. 3. The biochemical defect in rut: Adenylate cyclase activity in homogenates of normal (o) and rut (•) flies, subjected to centrifugation on a sucrose step gradient centrifugation. The soluble activity is on the left hand side; the particulate activity, which migrated into the interface between blocks of 20% and 60% sucrose, is much reduced in the mutant. The preparations were from abdomens, in which the rut defect is most pronounced. However, a defective particulate adenylate cyclase activity is clearly observed in all body regions. It appears that rut affects only one subpopulation (or functional state) of adenylate cyclase, whose relative proportion, out of the total cyclase activity, is larger in posterior body parts.

enzyme. However, in several systems, including brain and cells of neuronal origin, low concentrations of Ca^{2+} (<1 μM) stimulate adenylate cyclase activity (Brostrom et al., 1977). Such a biphasic response (i.e., stimulation at low Ca^{2+} and inhibition at high Ca^{2+}), was clearly detected by us (Dudai and Zvi, 1984) and by others (Livingstone et al., 1984) in normal adenylate cyclase but not in the rut enzyme. In other words, rut lacks at least a major proportion of the subpopulation (or functional state) of adenylate cyclase that is stimulated by Ca^{2+} (it should, however, be emphasized, as mentioned above, that rut cyclase is defective in other respects too, i.e., responsiveness to MgATP, Mg^{2+} and forskolin). Both the behavioral and

biochemical defects in rut were shown by Livingstone et al. (1984) to map to the same small segment of the X-chromosome, namely 12E1-13A5. Using small deletions of the X-chromosome, we have recently succeeded in narrowing down the rut locus to a smaller region spanning 12F (Zvi and Dudai, unpublished).

6. THE SEARCH FOR THE PHOSPHORYLATED SUBSTRATES

The role of cAMP in cells is to activate protein kinase, leading to phosphorylation of a protein substrate. Such phosphorylation underlies the physiological response of the cell to the signal that alters the level of cAMP. Since both dnc and rut have abnormal cAMP metabolism, it is pertinent to search for differences between protein phosphorylation in normal and mutant flies, with the hope of pinpointing protein substrate(s) that may be relevant to memory formation. The search for such substrates in Drosophila is currently being carried out by several laboratories. It is a very difficult task, since hundreds of proteins are phosphorylated in the tissue, given the appropriate conditions. The relevant proteins may be present in minute amounts and be phosphorylated or dephosphorylated very rapidly in a very specific microenvironment in vivo. The crucial proteins, the phosphorylation of which is necessary for normal memory and which are affected in dnc and rut, have not yet been identified, but ample information has been accumulated on the phosphorylation patterns in various preparations from Drosophila, both in vivo and in vitro; for example, differential rates of phosphorylation and dephosphorylation of various proteins can be demonstrated under different conditions (e.g., Fig. 4). Although such studies, suggesting dynamic equilibria between phosphorylated and dephosphorylated states of many Drosophila proteins, may corroborate working hypotheses as to the relevance of aberrations in cAMP metabolism to the behavioral defects (see below, and also Friedrich et al., 1986), we are still far away from identification of the phosphorylated substrates which are involved directly in the abnormal behavior.

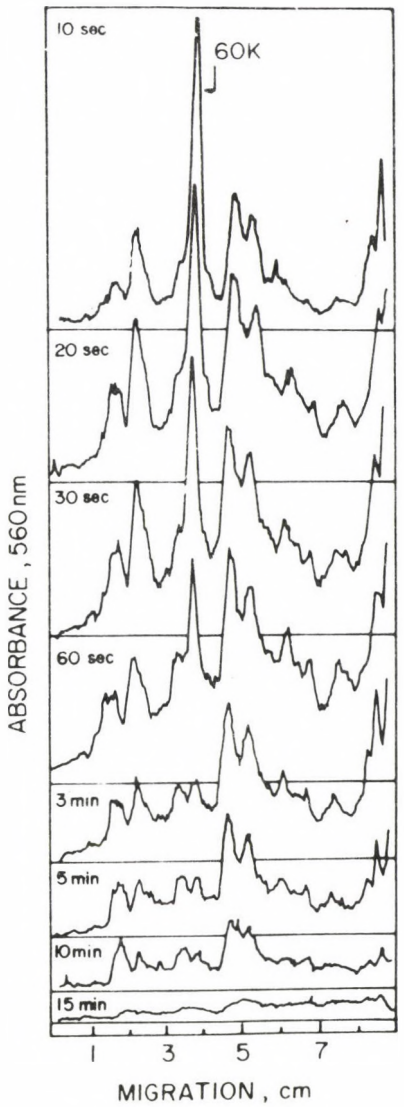


Fig. 4. Protein phosphorylation of Drosophila homogenates, as a function of incubation time with (γ-^{32}P)ATP. Reaction was carried out for the indicated times in the presence of 5 mM Mg^{2+} and 10^{-5} M cAMP. Samples were subjected to SDS-polyacrylamide electrophoresis on 10% polyacrylamide gel, and the developed autoradiograms were scanned. The figure depicts the densitograms thus obtained. It can be seen that the rate of phosphorylation and dephosphorylation of various bands is different, i.e. note decrease of ^{32}P content of the ca. 60K band already at 20 sec and the simultaneous increase in the adjacent bands. Adapted from studies by Uzzan (1981).

7. WHY DO THE MUTANTS FORGET?

dnc and *rut* implicate cAMP metabolism in memory formation in *Drosophila*. The gap between the mutated genes, the aberrant biochemistry and the abnormal behavior must, however, be bridged by studies on the cellular level, which would directly identify the role of each gene product in the physiological response. Studies of cellular components of a defined neuronal network that underlies a modifiable behavior are currently not feasible in *Drosophila*. Such studies are being carried out mainly in molluscs (Kandel and Schwartz, 1982; Neary et al., 1986), for which mutants are not available. It is tempting to combine the special advantages of the fruit fly and molluscs, and to correlate the results obtained with the first with the data and concepts that emerge from the study of the latters. Such a comparison indeed indicates that the results obtained with learning mutants of *Drosophila* fit quite nicely into a molecular model of learning that emerges from the study of molluscs (Fig. 5).

The studies of conditioning in *Aplysia* and *Hermissenda* can serve as prime examples. Studies on *Hermissenda* are described by Neary et al. (1986). We will therefore confine our attention here to the studies on the non-associative and associative modification of the gill-withdrawal reflex in *Aplysia* (Kandel and Schwartz, 1982). These studies in *Aplysia* indicate that cAMP-mediated phosphorylation of a K^+ channel underlies the activity-dependent amplification of presynaptic facilitation, which leads to an increased efficacy of information transfer in key synapses of the network that controls the behavior. Moreover, these studies also suggest that the mechanism of classical conditioning of the gill withdrawal reflex is an elaboration of the mechanism of the sensitization of the reflex; and that the pairing specificity, which is characteristic of the calssical conditioning, results from amplification of the facilitation by temporally paired spike activity in the presynaptic cells of the above mentioned key synapses (Hawkins et al., 1983; Walters and Byrne, 1983). It should be mentioned

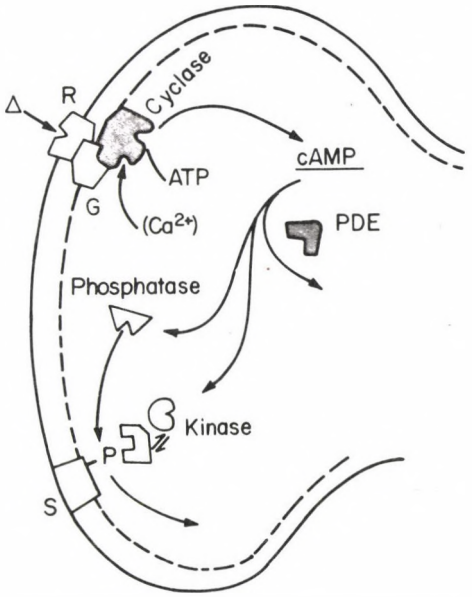


Fig. 5. A scheme of a hypothetical synaptic terminal, showing the enzymes which are defective in dnc (PDE, phosphodiesterase) and rut (adenylate cyclase). It is tempting to suggest that rut cannot form normal memory due to a lesion which is intimately associated with Ca^{2+}-stimulated adenylate cyclase. The defective enzyme may disrupt the information transfer via the receptor, R, from one cell to another in the learning network. In the case of dnc, abortive memory may be due to an increased cAMP level (a consequence of a defective PDE) which overshadows further increase in this second messenger; or to activation of specific phosphatases; or to conversion of an hypothetical substrate protein to a form that resists phosphorylation or favors dephosphorylation. In all the above cases, it is assumed that phosphorylation of a membranous substrate protein (S) is a key step in memory formation and prompt dephosphorylation erases this newly formed memory; for a comprehensive presentation of a molecular model of learning, on which these assumptions are based (see, Kandel and Schwartz, 1982).

that Drosophila mutants that affect conditioning also affect sensitization, suggesting that the two processes indeed share common mechanisms (Duerr and Quinn, 1982). Adenylate cyclase has been suggested to serve as the molecular locus for the pairing of the associative stimuli during the associative con-

ditioning in Aplysia; one working hypothesis suggests that the influx of Ca^{2+} with each action potential, interacts synergistically with a neurotransmitter-sensitive adenylate cyclase, so that the cyclase subsequently generates more cAMP in response to the neurotransmitter, provided that the arrival of the Ca^{2+} and the neurotransmitter occurs within the constraints of the required temporal pairing (Hawkins et al., 1983; Walters and Byrne, 1983). The findings on rut thus fit in such a model: the mutant seems to be defective in a Ca^{2+}-stimulated subpopulation of adenylate cyclase (Dudai and Zvi, 1984; Livingstone et al., 1984). rut may thus pave the way to the isolation of a macromolecule that links associative stimuli (Fig. 5).

The interpretation of the dnc results, in light of the above mentioned model, is more complicated. One possibility is that an increase in the level of cAMP which is induced by the relevant newly arrived signals in the appropriate synapses, is small compared to the chronically elevated cAMP levels. The new signals are therefore overshadowed. If this is the case, then dnc can be defined as an "information noise" mutant, since the postulated elevated noise in synapses prevents "printing" of novel information. Alternatively, one may suggest that the abnormally elevated cAMP levels favor molecular mechanisms that prevent, or erase, the chemical alterations that underlie memory formation. This could be due to a conversion of the hypothetical substrate protein into a form which resists phosphorylation, or to activation of phosphoprotein phosphatases that serve as memory erasers (Fig. 5). The intriguing data of Friedrich et al. (1986) and our preliminary results on normal flies, showing that phosphoproteins are differentially dephosphorylated in the presence of cAMP (Dudai, Uzzan and Buxbaum, unpublished), corroborate the latter possibilities. dnc may thus be a "memory erasure" mutant; this fits also with our behavioral data (Dudai, 1979; 1983). We are currently attempting to identify, both in bovine brain and in Drosophila brain, phosphoprotein phosphatases which may serve as the postulated "memory erasers".

In conclusion, two mutations in Drosophila implicate subpopulations of ubiquitous enzymes, which are involved in cAMP metabolism, in the mechanism of short-term memory. The results also fit into data obtained in studies on the cellular level in molluscs, and suggest that phosphorylation of yet unidentified polypeptides play a key role in the early phases of memory formation. Other Drosophila mutants may point in the future toward additional mechanisms of memory formation, including the consolidation of short-term into long-term memory.

Acknowledgements

The work carried out in our laboratory was supported by grants from the U.S.-Israel Binational Science Foundation, Jerusalem; The Fund for Basic Research administered by the Israeli Academy of Science, Jerusalem; The Israeli Ministry of Health, and the Forscheimer Center for Molecular Biology.

REFERENCES

Aceves-Pina, E.O., Booker, R., Duerr, J.S., Livingstone, M.S., Quinn, W.G., Smith, R.F., Sziber, P.P., Tempel, B.L., Tully, T.P. (1983) Cold Spring Harbor Symposium Quant. Biol. 48, 831-840

Brostrom, D.C., Brostrom, M.A., Wolff, D.J. (1977) J. Biol. Chem. 202, 5677-5685

Byers, D., Davis, R.L., Kiger, J.A. (1981) Nature, 289, 79-81

Dudai, Y. (1977) J. Comp. Physiol. 114, 69-89

Dudai, Y. (1979) J. Comp. Physiol. 130, 271-275

Dudai, Y. (1979) Molecular Mechanisms of Biological Recognition (M. Balaban, ed.), Elsevier, Amsterdam, pp. 341-

Dudai, Y. (1983) Proc. Natl. Acad. Sci. USA 80, 5445-5448

Dudai, Y., Jan, Y.N. (1975) California Institute of Technology Biology Annual Report p. 73

Dudai, Y., Zvi, S. (1984) Neurosci. Lett. 47, 119-124

Dudai, Y., Nahum-Zvi, S., Haim-Granot, N. (1980) Comp. Biochem. Physiol. 650, 135-138

Dudai, Y., Jan, Y.N., Byers, D., Quinn, W.G., Benzer, S. (1976) Proc. Natl. Acad. Sci. USA 73, 1684-1688

Dudai, Y., Uzzan, A., Zvi, S. (1983) Neurosci. Lett. 42, 207-212

Dudai, Y., Zvi, S., Segel, S. (1984) J. Comp. Physiol. (in press)

Duerr, J.S., Quinn, W.G. (1982) Proc. Natl. Acad. Sci. USA 79, 3646-3650

Friedrich, P., Dévay, P., Solti, M., Pintér, M. (1986) Acta Biochim. Biophys. Hung. 21, 193-203

Hawkins, R.D., Abrams, T.W., Carew, T.J., Kandel, E.R. (1983) Science 219, 400-405

Kandel, E.R., Schwartz, J.H. (1982) Science 218, 433-443

Livingstone, M.S., Sziber, P.P., Quinn, W.G. (1984) Cell 37, 205-215

Neary, J.T., Alkon, D.L. (1986) Acta Biochim. Biophys. Hung. 21, 159-176

Quinn, W.G., Dudai, Y. (1976) Nature 262, 576-577

Quinn, W.G., Harris, W.A., Benzer, S. (1974) Proc. Natl. Acad. Sci. USA 71, 708-712

Schramm, M., Selinger, Z. (1984) Science 225, 1350-1356

Uzzan, A. (1981) M.Sc. Thesis, The Weizmann Institute, Rehovot

Walters, E.T., Byrne, J.H. (1983) Science 219, 405-408

Acta Biochim. Biophys. Hung. 21(3), pp. 193-203 (1986)

PROTEIN PHOSPHORYLATION IN *DUNCE* MEMORY-MUTANT DROSOPHILA*

Peter Friedrich, Piroska Dévay, Magda Solti and Marianna Pintér

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, P.O. Box 7, H-1502, Hungary

(Received May 15, 1985)

The *dunce* mutant strains of *Drosophila melanogaster* are defective in associative learning (Dudai et al., 1976; Acaves-Pina and Quinn, 1979; Tempel et al., 1983) and non-associative learning (Duerr and Quinn, 1982), as well as in female fertility (Mohler, 1977). Furthermore, they have diminished activity of a cAMP-phosphodiesterase (PDE-II), one of the two major cyclic nucleotide-phosphodiesterase isoenzymes present in wild type flies, with consequentially elevated cAMP levels (Davis and Kiger, 1981; Byers et al., 1981). In all other respects the *dunce* flies seem to be normal (Dudai et al., 1976). Evidence has been put forward that the *dunce* mutation is located in the structural gene of PDE-II (Kauwar, 1982; Shotwell, 1983) and that PDE-II is present in normal, but absent from amorphic *dunce*, Drosophila brain (Solti et al., 1983). Behavioral studies revealed that *dunce* flies are affected in memory retention rather than acquisition: mutants learn practically as well as wild type flies, but their memory rapidly vanes (Dudai, 1979; 1983). It is generally postulated that the impaired memory of *dunce* flies is due to the anomalous cAMP metabolism.

*Paper presented at the round table discussion on "Protein Phosphorylation and Memory Mechanisms" organized in the frame of the 5th Meeting of the European Society for Neurochemistry, Budapest, August 21-24, 1984.

Akadémiai Kiadó, Budapest

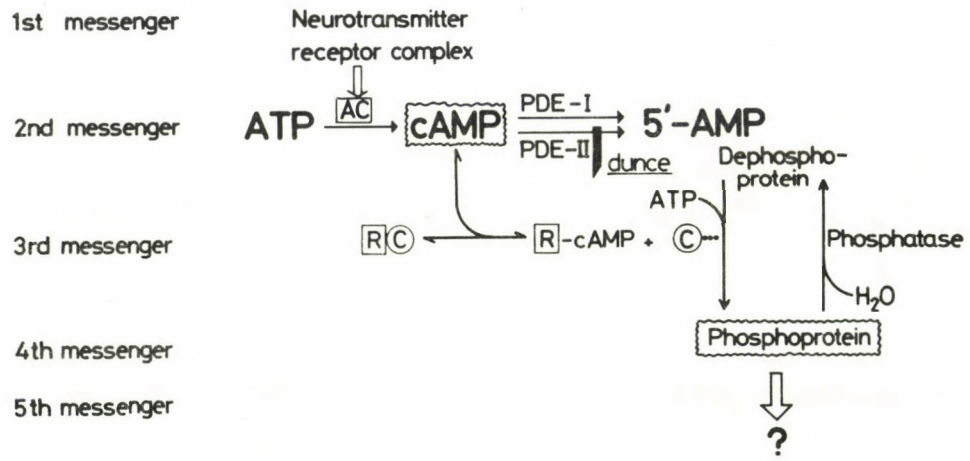


Fig. 1. Organizational levels in the protein phosphorylation cascade and its shift in the dunce mutant of Drosophila. AC, adenylate cyclase; PDE, cyclic nucleotide phosphodiesterase; R and C, the regulatory and catalytic, subunits, respectively, of cAMP-dependent protein kinase. Wavy frames denote elevation in dunce. The identity, function and target of the phosphoprotein(s) are unknown.

The scheme of events expected to occur in the pertinent neurons of dunce flies is quite straightforward, up to a certain point (Fig. 1). Knocking out of PDE-II by the dunce mutation causes an increase of cAMP level, which may attain particularly high, protracted peaks when adenylate cyclase is activated by neurotransmitters. As the main, if not sole, target of cAMP is the regulatory (R) subunit of cAMP-dependent protein kinase, the likely effect is an enhanced dissociation and activation of this kinase, which in turn results in a greater degree of phosphorylation of various substrate proteins. However, at this point, which after Greengard (1984) may be referred to as fourth messenger level, our ignorance becomes complete. What gets phosphorylated and how does it lead to the behavioural deficit?

While the answers to these questions are still remote, some basic predictions of the model could already be tested. One was the enhanced activity of cAMP-dependent protein kinase in dunce. The direct approach, i.e. the quantitation of free catalytic (C) subunit in vivo, would be fraught with error because after cell disruption secondary redistributions take

place. Some years ago we devised a technique by the aid of which the concentration in vivo of a protein-ligand complex could be assessed, at that time in human erythrocyte metabolism (Solti and Friedrich, 1979). This "enzyme-probe" method consists of the monitoring of the decay of a metabolite by a degradative enzyme in a fresh homogenate and from the kinetics of decay one can tell the amount of free and (protein)-bound metabolite, because binding usually protects the metabolite from degradation. This approach seemed applicable to the R subunit-cAMP complex (Friedrich et al., 1984).

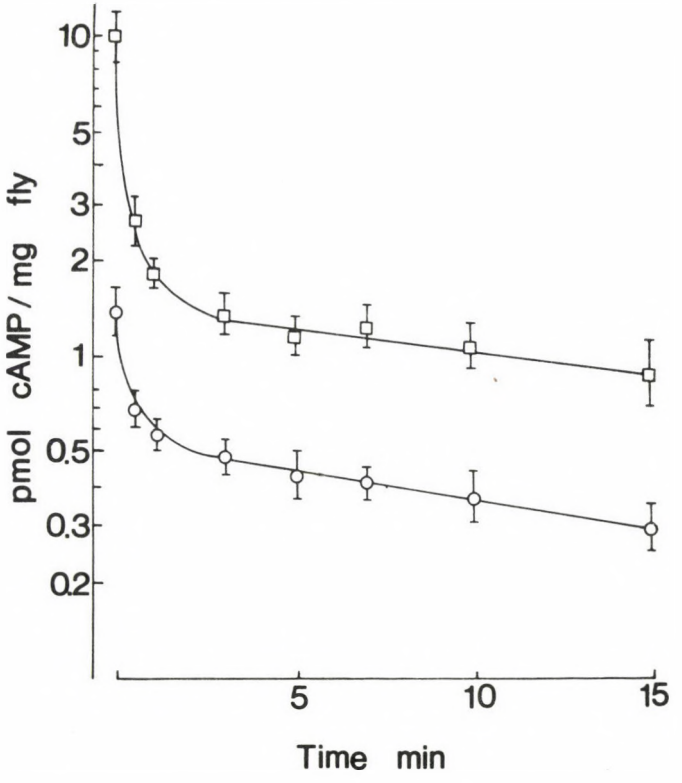


Fig. 2. Time course of cAMP decay in homogenates of Canton-S and dunce^{M11} flies. The homogenate (100 mg fly/ml) was made and incubated at 0°C; at the times indicated samples were taken for cAMP radioummunoassay. The mean ±SD of seven independent experiments is shown for both Canton-S (o) and dunce^{M11} (□). From Friedrich et al. (1984)

Fig. 2 shows the decay of cAMP in fresh Drosophila homogenates for wild type Canton-S and dunce^{M11} flies. This mutant strain is amorphic with respect to PDE-II and its cAMP content is about six times the normal. The decay curves in Fig. 2 are biphasic: the initial rapid phase corresponds to free cAMP, whereas the slow phase can be assigned to bound nucleotide. Namely, control experiments have shown that in the slow phase of cAMP breakdown the rate-limiting step is the dissociation of nucleotide from a binding species rather than enzymatic hydrolysis per se. As seen in Fig. 2, the slow phases in the two strains have about the same apparent first order rate constant but the ordinate intercepts, which give the amount of the bound species, are different: the bound cAMP fraction in dunce^{M11} is about three times as high as in the wild type (1.5 vs. 0.55 pmoles/mg fly). The most plausible candidate for a binding protein is the R subunit of cAMP-dependent protein kinase. This means then that in dunce^{M11} the R subunit-cAMP complex concentration is elevated, which in a first approximation implies a higher free C subunit concentration. An alternative interpretation would be that in dunce there is a higher overall R subunit concentration, presumably to buffer, at least in part, the increased cAMP level.

Another prediction of the above model is that there should be some extra phosphorylation in dunce compared with the wild type. We have tested this (Dévay et al., 1984) by feeding normal and mutant flies with $^{32}P_i$, then the heads were homogenized and analyzed by SDS-polyacrylamide gel-electrophoresis followed by autoradiography. Fig. 3 shows the densitometric tracings of the patterns. In the two dunce alleles examined (dunce^{2} and dunce ^{M11}) labelling increased relative to the normal especially around 120 kD apparent molecular mass, and this gradual increase roughly parallels the elevation in the cAMP content of the strains. Even at this low resolution obtained by one-dimensional electrophoresis, we find support for the working hypothesis that dunce strains exhibit somewhat enhanced protein phosphorylation.

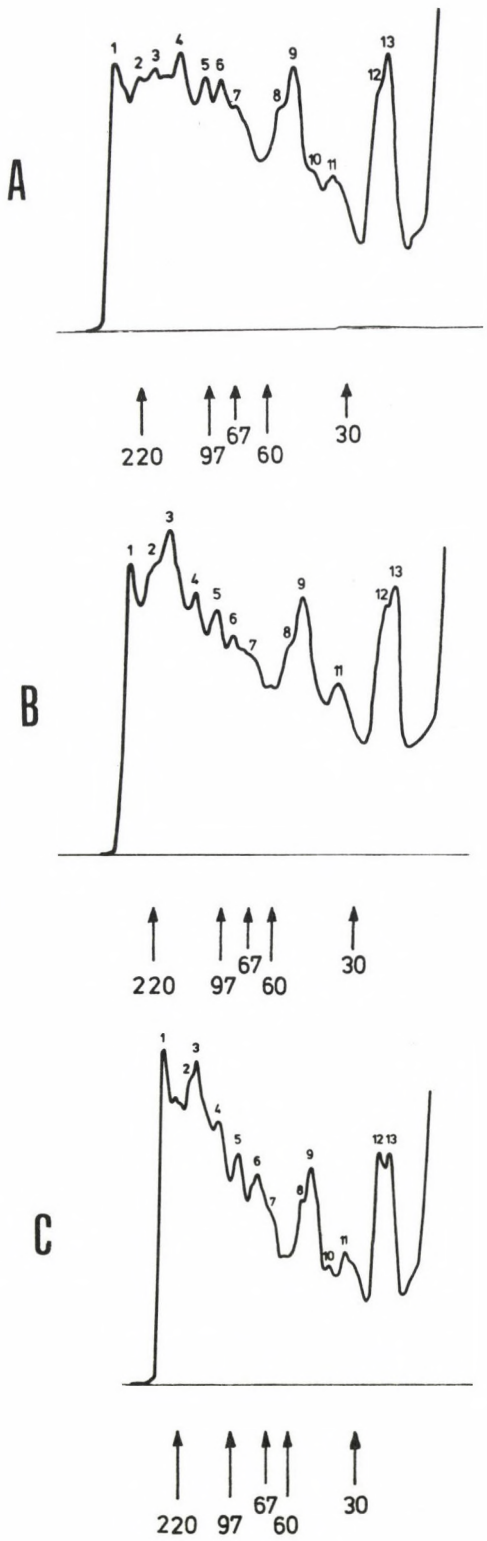


Fig. 3. Protein phosphorylation patterns of Drosophila heads after labelling with $^{32}P_i$ *in vivo*. Heads were homogenized and subjected to SDS-gel electrophoresis followed by autoradiography. The densitometric tracings are shown: A, Canton-S; B, *dunce*^{2}; C, *dunce*^{M11}. The positions of molecular weight markers, in kilodalton, are indicated. From Dévay et al. (1984).

In cell-free system (fresh fly head homogenate) we also found some characteristic differences when protein phosphorylation was run with $|\gamma\text{-}^{32}P|$ATP. Beside the high molecular mass region, there was a definite increase of labelling in a band at 53 kD in <u>dunce</u>^{M11} (Fig. 4). Addition of cAMP to the homogenate markedly increased the intensity of this band; qualitatively the same result was obtained with the PDE inhibitor, theophylline. In contrast, cGMP was much less effective and Ca^{2+}/calmodulin was without any effect on the 53 kD band. These data suggest that the 53 kD polypeptide is a substrate for cAMP-dependent protein kinase.

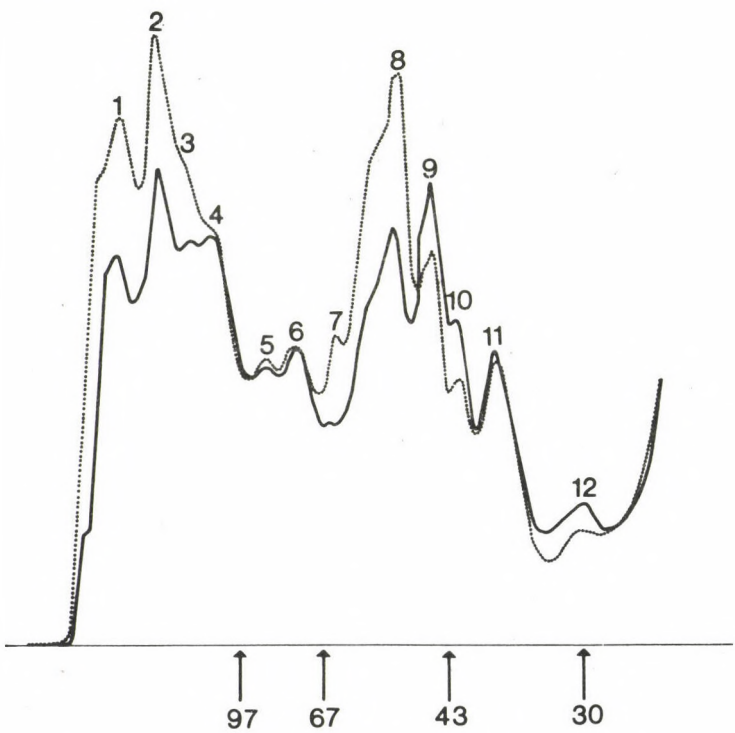


Fig. 4. Protein phosphorylation patterns of Canton-S and <u>dunce</u>^{M11} fly heads after labelling in homogenate with $|\gamma\text{-}^{32}P|$ATP <u>in vitro</u>. Continuous line: Canton-S; <u>dotted line</u>: <u>dunce</u>^{M11}. The molecular weight markers are <u>indicated</u>.
From Dévay et al. (1984).

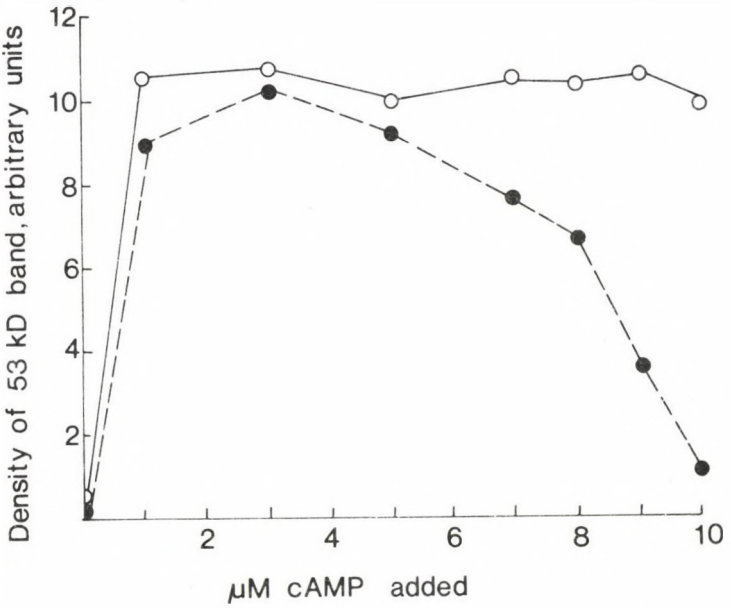


Fig. 5. Effect of cAMP concentration on the labelling of 53 kD protein in vitro. Phosphorylation time: 3 min, at 0°C, in fly head homogenates. Density, measured on the autoradiograms of dried SDS-gel slabs, is in arbitrary units. o, Canton-S; ●, dunce^{M11}. From Dévay et al. (1986).

When studying the dependence of labelling of the 53 kD band on cAMP concentration a peculiar difference was observed between wild type and dunce^{M11} flies. While in the former 1 to 10 μM cAMP added to the homogenate produced about the same extent of labelling, in the latter labelling dramatically decreased towards higher cAMP concentrations in the same range (Fig. 5). Such a decline of labelling may stem either from decreased phosphorylation or increased dephosphorylation. To decide which was the case, the time course of labelling of the 53 kD band was followed (Fig. 6). While the label was nearly constant in Canton-S over the time period examined, with dunce^{M11} it decreased in time from the earliest possible measurement onwards. Importantly, the decrease of label could also be elicited in Canton-S, if theophylline was included in the reaction mixture. It appears that the observed phenomenon

is enhanced protein dephosphorylation induced by an appropriately high cAMP concentration.

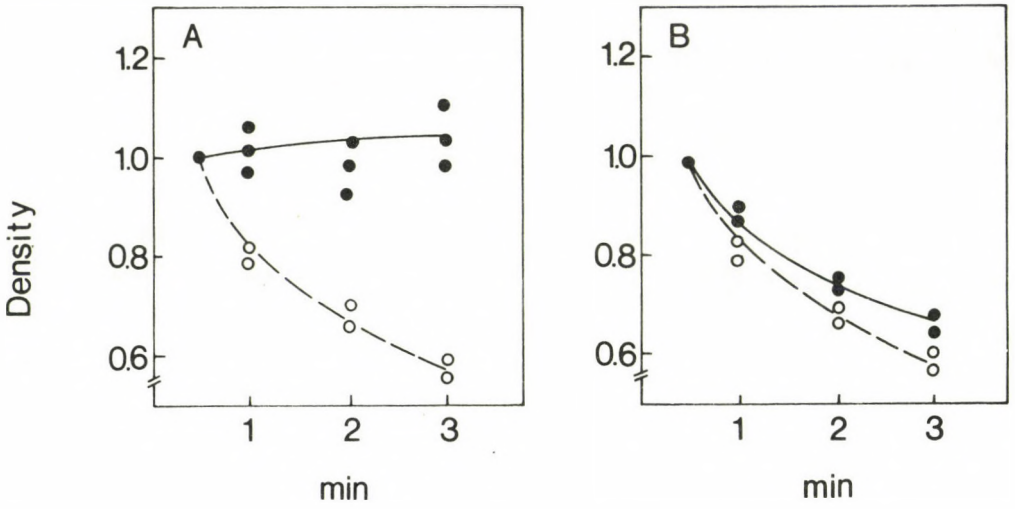


Fig. 6. Time course of labelling of the 53 kD band-protein *in vitro*. Labelling was evaluated by densitometry of the autoradiograms made of the dried SDS-gel slabs. The density measured in the first point (30 sec) was taken as unity. A, Canton-S head homogenate with 10 µM cAMP (●) and with 10 µM cAMP + 10 mM theophylline (o); B, *dunce*^{M11} head homogenate with 10 µM cAMP (●) and with 10 µM cAMP + 10 mM theophylline (o). From Dévay et al. (1986).

In the case of the marine snails Aplysia and Hermissenda, where behaviour can be correlated with biochemistry at the level of individual neurons, one of the key events in both associative and non-associative learning is the phosphorylation of a putative potassium channel protein (Kandel and Schwartz, 1982; Alkon et al., 1982; 1983; Hawkins et al., 1983; Neary and Alkon, 1983). The decay of memory must then involve the removal of the phosphoryl group(s), i.e. it is a dephosphorylation process. It is tempting to draw a formal analogy between mollusks and Drosophila. If a similar mechanism is operative in the fruit fly, then the rapid vanishing of memory in *dunce* may be effected by enhanced dephosphorylation in certain neurons. Above we described an apparently analogous

phenomenon: the high cAMP level in dunce flies elicited the rapid dephosphorylation of a 53 kD protein.

This analogy, however, must be regarded with caution. The protein undergoing rapid dephosphorylation in dunce flies very probably does not belong to a potassium channel, but seems to be identical with the R subunit of cAMP-dependent protein kinase (Dévay et al., 1986). If so, our experiments reflect a particular, rather than general, dephosphorylation enhancement. Nevertheless, in light of recent advances indicating kinase-induced activation of a protein phosphatase (Resink et al., 1983; Jurgensen et al., 1984) we cannot rule out a priori the existence of phosphatase(s) that are stimulated directly or indirectly by cAMP.

It is pertinent to mention that the rapid decay of memory is also observed in the Drosophila mutant rutabaga, which has slightly diminished cAMP levels as compared to the wild type, owing to the apparent lack of a Ca^{2+}/calmodulin activated adenylate cyclase catalytic subunit (Dudai et al., 1983; Dudai and Zvi, 1984; Livingstone et al., 1984). However, there is no reason to believe that in a system as complex as the one involved in cAMP metabolism and protein phosphorylation, the same gross functional alteration can only be achieved in a single way. In fact, the memory deficiencies in dunce and rutabaga mutants are not quite the same, as detected by careful behavioural studies (Dudai, 1983), suggesting at least in part different molecular mechanisms.

The biochemical work on memory-mutant Drosophila described above refers to whole heads rather than to the very cells involved in the learning process. Evidently, both inter- and intracellular compartmentations play a pivotal role in the specific reactions of the nervous system. Components barely detectable when analyzed en masse may be crucial in a particular reaction sequence confined to a small group of neurons. This is how, for example, the deficiency in the Ca^{2+}-activation of adenylate cyclase in the mutant rutabaga can be conceived as relevant to memory, even though the deficiency is most pronounced in the abdomen (Livingstone et al., 1984). On the

other hand, a phenomenon as robust as the phosphorylation-dephosphorylation of the 53 kD protein observed by us in the fly head homogenates need not occur or have the same functional consequences in all cells.

In order to get meaningful answers about the memory mechanisms of Drosophila, the genetic and biochemical dissection ought to be extended to the enzymes and substrates of protein phosphorylation, invoking also the techniques of molecular genetics.

REFERENCES

Aceves-Pina, E.Q. and Quinn, W.G. (1979) Science 206, 93-96

Alkon, D.L., Acosta-Urquidi, J., Olds, J., Kuzma, G. and Neary, J.T. (1983) Science 219, 303-306

Alkon, D.L., Lederhandler, I. and Shoukimas, J. (1982) Science 213, 693-695

Byers, D., Davis, R.L. and Kiger, J.A., Jr. (1981) Nature 289, 79-81

Davis, R.D. and Kiger, J.A., Jr. (1981) J. Cell Biol. 90, 101-107

Dévay, P., Solti, M., Kiss, I., Dombrádi, V. and Friedrich, P. (1984) Int. J. Biochem. 16, 1401-1408

Dévay, P., Pintér, M., Yalcin, A.S. and Friedrich, P. (1986) Neuroscience 18, 193-208

Dudai, Y. (1979) J. Comp. Physiol. 130, 271-275

Dudai, Y. (1983) Proc. Natl. Acad. Sci. USA 80, 5445-5448

Dudai, Y., Uzzan, A. and Zvi, S. (1983) Neurosci. Lett. 42, 207-212

Dudai, Y. and Zvi, S. (1984) Neurosci. Lett. 47, 119-124

Dudai, Y., Yan, Y.N., Byers, D., Quinn, W.G. and Benzer, S. (1976) Proc. Natl. Acad. Sci. USA 73, 1684-1688

Duerr, J.S. and Quinn, W.G. (1982) Proc. Natl. Acad. Sci. USA 79, 3646-3650

Friedrich, P., Solti, M. and Gyurkovics, H. (1984) J. Cell Biochem. 26, 197-203

Greengard, P. (1984) Proc. Fifth Internat. Conf. on Cyclic Nucleotides and Protein Phósphorylation, Raven Press, New York

Hawkins, R.D., Abrams, T.W., Cares, T.J. and Kandel, E.R. (1983) Science 219, 400-405

Jurgensen, S., Shackter, E., Huang, C.Y., Chock, P.B., Yang, S.D Vandenheede, J.R. and Merlevede, W. (1984) J. Biol. Chem. 259, 5864-5870

Kandel, E.R. and Schwartz, J.H. (1982) Science 218, 433-443

Kauvar, L.M. (1982) J. Neurosci. 2, 1347-1358

Livingstone, M.S., Sziber, P.P. and Quinn, W.G. (1984) Cell 37, 205-215

Mohler, J.D. (1977) Genetics 85, 259-272

Neary, J.T. and Alkon, D.L. (1983) J. Biol. Chem. 258, 8979-8983

Resink, T.J., Hemmings, B.A., Tung, H.Y.L. and Cohen, P. (1983) Eur. J. Biochem. 133, 455-461

Shotwell, S.L. (1983) J. Neurosci. 3, 739-747

Solti, M., Dévay, P., Kiss, I., Londesborough, J. and Friedrich, P. (1983) Biochem. Biophys. Res. Commun. 111, 652-658

Solti, M. and Friedrich, P. (1979) Eur. J. Biochem. 95, 551-559

Tempel, B.L., Bonini, N., Dawson, D.R. and Quinn, W.G. (1983) Proc. Natl. Acad. Sci. USA 80, 1482-1486

Acta Biochim. Biophys. Hung. 21(3), pp. 205-214 (1986)

THE EFFECT OF CHRONIC ALCOHOL INGESTION ON THE CONTRACTILE PROTEINS OF THE RAT HEART

István Édes[*§], Ödön Takács[+], Miklós Csanády[*], Ferenc Guba[+]

2nd Department of [*]Medicine, Division of Cardiology and Department of [+]Biochemistry, University Medical School, Szeged, Hungary

(Received June 5, 1984)

SUMMARY

With increasing duration of alcohol consumption the amounts of total myofibrillar proteins in CFY rats decreased slightly, but significantly (from 63.3±5.7 $mg.g^{-1}$ wet muscle weight to 54.9±5.9 $mg.g^{-1}$ after 12 weeks on alcohol). Similar slight changes could be observed in the case of sarcoplasmic proteins. No significant changes were observed in the composition of the myofibrillar proteins. The densitiometrically calculated percentage ratios of myosin/actin, myosin light chains (LC_1/LC_2) and troponin components remained the same in the alcoholic animals. The same distribution of native myosin isoenzymes was found in the ventricles of the alcoholic animals as in the controls. We found no electrophoretically detectable evidence that a change in the composition of the myofibrillar proteins is responsible for the decreased contractility of the rat heart following chronic alcohol ingestion.

INTRODUCTION

For more than 100 years considerable evidence has been accumulating of an association between excessive alcohol consumption and heart disease in man (Bollinger, 1884). In the past 20 years it has become evident that alcohol impaire contraction function, fine structure and metabolism of the myocardium (Alexander, 1967; Bulloch et al., 1972; Burch and

[§]Address for correspondence: István Édes, 2nd Department of Medicine, University Medical School, 6701 Szeged, P.O. Box 480, Hungary

Akadémiai Kiadó, Budapest

Walsh, 1960; Burch and DePasquale, 1969; Édes et al., 1983; Wendt et al., 1965). Noninvasive (Spodick et al., 1972) and invasive (Regan et al., 1969) studies have shown reduced contractility of the left ventricle in alcoholic subjects, either with or even without symptoms or clinical evidence of heart disease or malnutrition.

However, the pathogenesis of alcohol-induced cardiac lesions has not been clearly established. It has been shown that rat cardiac ventricular myosin is composed of three different isoenzymes, referred to as V_1, V_2 and V_3 (Hoh et al., 1979). The proportions of these isoenzymes change during ontogenic development and in certain pathogenic conditions. It has been suggested (Schwartz et al., 1981) that myosin isoenzymatic variations constitute an adaptive alteration of the myocardial cell, which contributes to new functional requirements.

The present study was undertaken in an attempt to determine the effect of chronic alcohol ingestion on the contractile proteins, to acquire a more complete understanding of the pathogenesis of alcoholic cardiomyopathy.

METHODS

General

Studies were performed on 48 CFY rats weighing 150 to 160 g. They were randomly assigned to three groups (A, B and C) and fed as follows: Group A (12 rats) were given a laboratory stock diet and separately a solution of 6.95 M alcohol and 0.73 M sucrose in tap-water, according to Ports and Gomez (1968), for 6 weeks. Members of Group B (12 rats) received the same stock diet and alcohol + sucrose mixture for 12 weeks. Group C (24 rats) were provided with a laboratory stock diet and 0.73 M sucrose solution for 6 or 12 weeks. The rats of Group C were fed their diet in quantities sufficient to result in a growth curve matching that of the alcoholics. This group was regarded as the control for Groups A and B. The alcohol in the diet axxounted for 40 to 45% of the total calorie intake. After 6 weeks on test, 12 experimental and 12 contol rats were killed by exsanguination under light ether anesthesia. After

12 weeks the remaining alcoholic and control animals were killed in the same manner. No significant changes in myocardial protein contents were found between the 6 and 12-week control groups, and the results of the two groups were combined in the protein determinations. The thoracic cavity was opened and the still beating heart was removed, cleaned, washed and weighed. The biochemical examinations were made on the isolated left ventricle.

Fractionation of proteins

Homogenization was performed in 10 volumes of 0.13 M KCl, 50 mM phosphate buffer (pH 7.4). Proteins were fractionated according to Takács et al. (1977). With this method only negligible amounts of proteins were lost. Protein contents were determined by the Peterson (1977) technique, with bovine serum albumin as standard. No significant difference in myocardial water content was found between the alcoholic and control groups. The determined protein values are given as $mg.g^{-1}$ wet muscle weight.

Myosin isolation and digestion procedures

Crude extraction of myosin without further purification was carried out according to d'Albis et al. (1979). The electrophoretic separation of the native myosin isoenzymes in this crude preparation was satisfactory and the samples did not need further purification.

The partial proteolytic cleavage of sodium dodecyl sulfate (SDS)-denatured myosins with Staphylococcus aureus protease was carried out as described by Cleveland et al. (1977). The proteolytic products were further analysed on a slab gel composed of 12% (w/v) polyacrylamide.

Electrophoretic procedures

Electrophoresis under nondenaturing conditions was carried out on cylindrical gel tubes by pyrophosphate polyacrylamide gel electrophoresis, as described by Hoh et al. (1978).

Electrophoresis in the presence of SDS was performed on 7 or 12% polyacrylamide slab gels according to Talbot and Yphantis (1971). The gels were stained with 0.04% (w/v)

Coomassie blue. The individual components were identified through the co-electrophoresis of selectively extracted and purified reference proteins. The individual bands were evaluated with a Kipp and Zonen densitometer equipped with an integrator. From the different known affinities of the proteins to Coomassie blue, the densitometrically calculated distribution was corrected in accordance with the staining index of each myofibrillar protein. The latter were determined by means of purified reference proteins which were free from electrophoretically detectable contaminants.

Statistics

The results were submitted to statistical evaluation with Student's t test. Values with $P<0.05$ were regarded as statistically significant.

RESULTS

Animal body and heart weights

The mean body weights of the alcoholic animal were 154±5 g at the beginning of the experiment, and 205±8 g and 256±10 g after 6 and 12 weeks, respectively. The body weights of the controls (Group C) did not differ significantly from those of the alcoholics.

When killed, the alcoholic animals exhibited slight, but significant cardiomegaly, with dilated atrial and ventricular chambers. The heart/body weights ratio was significantly less in the controls (2.48±0.12 $g.kg^{-1}$) than in the alcoholics (2.69±0.10 and 2.87±0.15 $g.kg^{-1}$ in Groups A and B, respectively).

Changes in the myofibriller and sarcoplasmic protein fractions

In the myocardium of the control animals the myofibrillar protein content was 63.3±5.7 $mg.g^{-1}$ wet muscle weight. With increasing duration of alcohol consumption the amount of myofibrillar proteins gradually decreased in the alcoholic animals (Fig. 1). The decrease was slight, but significant after 12 weeks. Similar slight significant changes could be observed in the case of the sarcoplasmic proteins. A slight increase was

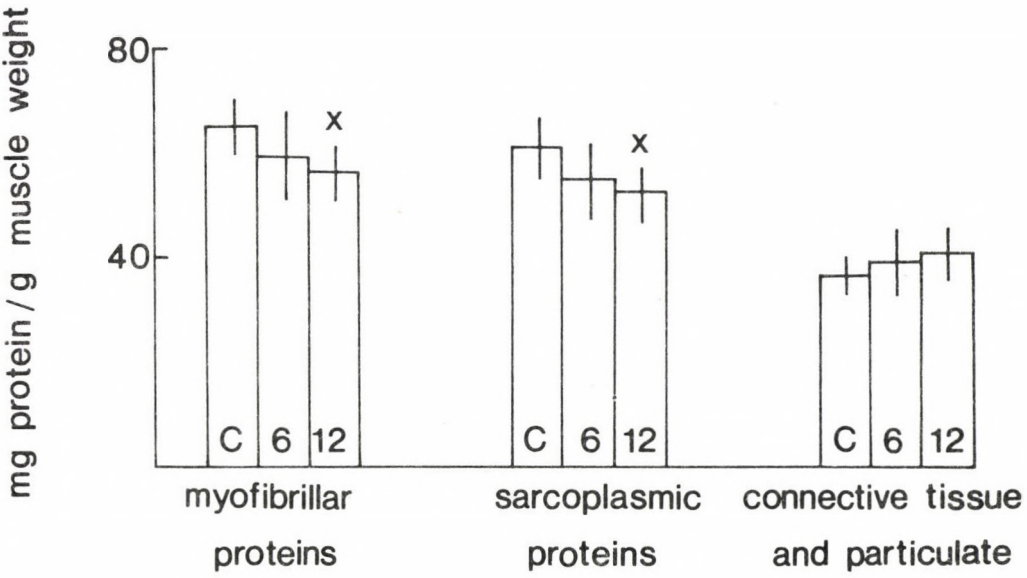


Fig. 1. Effect of chronic alcohol ingestion on protein distribution in cellular components. Numbers in columns indicate the duration of alcohol consumption in weeks. C stands for the control. X denotes significant changes ($P<0.05$) compared with the control.

found in the protein content of the "connective tissue + particulate" fraction as a result of alcohol ingestion. However, the increase was not statistically significant even after 12 weeks on alcohol ($0.1>P>0.05$).

Electrophoresis of myofibrillar proteins and native myosins

Table 1 shows the densitometrically calculated distribution of myofibrillar proteins. The major myofibrillar components of the cardiac muscle, which is made up almost exclusively of slow-oxidative fibers, are as follows: myosin heavy chain, actin, tropomyosin, troponins and myosin light chains. Samples from the ventricles of alcoholic animals, no new bands were seen to suggest either the occurence of abnormal proteins or proteolytic cleavage of normal proteins. In both the alcoholic and the control animals the ratio of myosin/actin is about 4.8. This value was not changing during the experimental period. The proportions of myosin light chains and of troponin

Table 1. Percentage distribution of contractile proteins in the myocardium of control and alcoholic rats

	M.W. ($x10^3$)	Control	6 weeks on alcohol	12 weeks on alcohol
1. myosin heavy chain	200	47.4±2.8	47.4±3.0	48.0±3.1
2. M proteins (α and β chains) C proteins α actinin		9.3±1.8	9.9±1.5	9.7±1.6
3. actin	45	12.7±1.2	12.8±1.2	12.9±1.3
4. troponin-T	41	3.6±0.8	3.3±0.8	3.2±0.7
5. tropomyosin	38	4.9±0.9	4.6±0.8	4.5±1.0
6. troponin-I	32	2.7±0.5	2.6±0.5	2.5±0.6
7. unknown-1	30	2.1±0.7	2.1±0.7	1.9±0.6
8. myosin LC_1	25	6.4±0.8	6.6±0.9	6.5±0.8
9. unknown-2	24	1.2±0.3	1.1±0.4	1.0±0.3
10. troponin-C	19.5	2.6±0.5	2.5±0.5	2.4±0.4
11. myosin LC_2	17.5	7.1±1.0	7.1±0.9	7.4±1.1

Mean±SD; M.W. indicates the apparent molecular weights.
The densitometrically calculated distribution was corrected in accordance with the staining index. For further details, see the methods.

components remained the same in the alcoholic animals as in the controls.

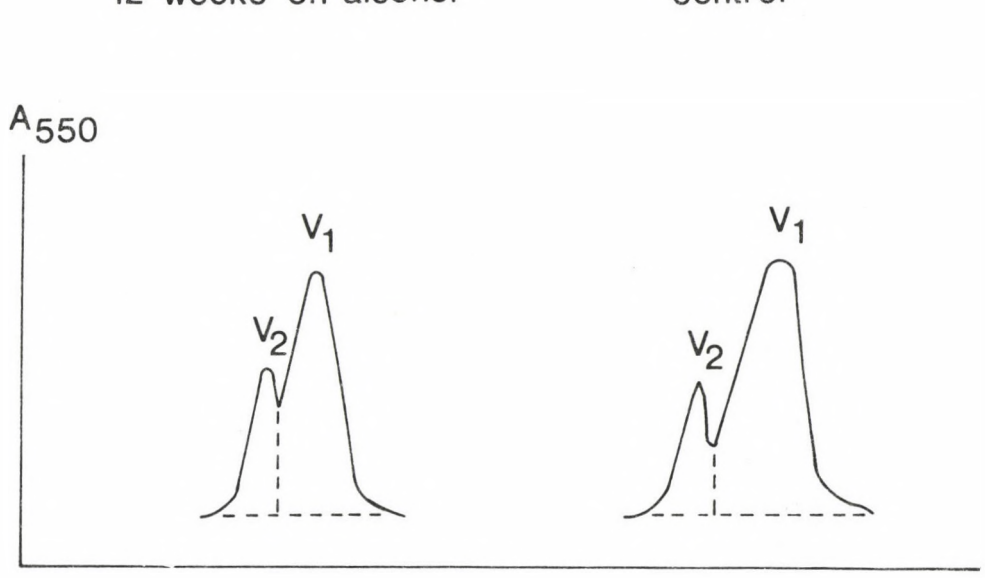


Fig. 2. Representative pyrophosphate gel patterns (inserts) and representative densitometric scans of isoenzyme gels for the control and alcoholic groups.
V_1 and V_2 refer to the myosin isoenzymes.

The electrophoretic pattern of native myosins from the rat heart ventricles is shown in Fig. 2. The ventricle of adult, control animals contained mostly V_1, with a slight amount of V_2. The densitometrically calculated percentage distribution of isoenzymes was as follows: V_1, 80.3±4.5; V_2, 19.7±1.8%; V_3 could be detected only in trace amounts in the overloaded gel electrophoretic samples. The same distribution of native myosin isoenzymes could be observed in the ventricles of alcoholic animals. This was confirmed by the partial Staphylococcus aureus protease digestion of SDS-treated myosin heavy chains. The one-dimensional analysis of the complex mixture of polypeptides produced by digestion of the heavy chains did not reveal any qualitative difference between the alcoholic and control groups.

DISCUSSION

Excessive and prolonged consumption of alcoholic beverages leads to deterioration of the cardiac function, which may progress to "alcoholic cardiomyopathy" and ultimately to cardiac arrest or congestive heart failure. There appears to be a consensus that the effects of alcohol are multifocal and complex, but finally always lead to critically reduced contractility of the left ventricle. Even the metabolites of alcohol are toxic for the myocardium, since Schreiber et al. (1972) pointed out that acetaldehyde is a more potent inhibitor of myocardial protein synthesis than ethanol per se.

In our experiment we have shown that prolonged alcohol consumption results in only slight quantitative changes in the myofibrillar and sarcoplasmic protein contents of the heart muscle of the rat. The observed shifts in the protein fractions of subcellular components may biochemically reflect the electronmicroscopic findings (disintegrated myofibrils, large mitochondria, interstitial fibrosis, focal atrophy) in alcoholic cardiomyopathy (Alexander, 1976; Bulloch et al., 1972; Mall et al., 1980).

Pronounced decreases in the contractile proteins have been reported in the skeletal muscles following denervation (Takács et al., 1974), immobilization (Takács et al., 1981) and muscular dystrophies (Brust, 1966). A prolonged lack of activity results in changes in the peptides participating in the contraction-relaxation regulation. However, the reaction of the skeletal muscles depends on their function.

In the case of heart, the tissue can only slightly be influenced to study the adaptation of the muscle to new functional requirements. Changes in the myosin isoenzyme distribution, i.e. an increase in the V_3 isoenzyme form, have been described in the rat heart during aging (Hoh et al., 1978), after mechanical overload (Scheuer et al., 1982; Schwartz et al., 1981), in experimental diabetes (Dilman, 1980) and in hypothyroidism (Hoh et al., 1978; Schwartz et al., 1982). Recently, Wiegand et al. (1983) reported similar changes in

the myosin isoenzyme distribution in the genetically cardiomyopathic Syrian hamster. In these pathologic conditions (except for the fetal stage), in contrast to the heavy chains no changes could be observed in the light chains (Schwartz et al., 1982). However, Schaub et al. (1984) reported that in human congestive cardiomyopathies, probably owing to alcohol consumption, the atrial light chain $LC_{1\ atrial}$ appeared in the left ventricle in 11.7% of the investigated cases.

Prolonged alcohol consumption in rats slightly decreased the myofibrillar protein content of the myocardium, but did not cause any electrophoretically detectable qualitative difference as compared to the controls. No changes could be observed in the myosin isoenzyme distribution or light chain pattern of the alcoholic animals. Though the electrophoretic densitometric readings show only relative proportions of proteins and minor changes in the myofibrillar components can not be excluded completely, it seems unlikely that primary alterations in contractile proteins would be responsible for the final pathophysiological picture following chronic alcohol ingestion.

Acknowledgements

This work was supported by the Hungarian Ministry of Health (4.02-0306-01-1). We are grateful for the helpful technical advice of Dr Margit Keresztes and the technical assistance of Dr Ágota Andó, Mrs Éva Tóth and Miss Zita Felhő.

REFERENCES

d'Albis, A., Pantaloni, C., Bechet, J.J. (1979) Eur. J. Biochem. 99, 261

Alexander, C.A. (1967) Br. Heart J. 29, 200

Bollinger, O. (1884) Deutsche med. Wchschr. 10, 180

Brust, M. (1966) Am. J. Physiol. 210, 445

Bulloch, R.T., Pearce, M.B., Murphy, M.L., Jenkins, B.J., Davis, J.L. (1972) Am. J. Cardiol. 29, 15

Burch, G.E., Welsh, J.J. (1960) Am. J. Cardiol. 6, 864

Burch, G.E., DePasquale, N.P. (1969) Am. J. Cardiol. 23, 723

Cleveland, D.W., Fisher, S.G., Kirschner, M.W. (1977) J. Biol. Chem. 252, 1102

Dilman, W.H. (1980) Diabetes 29, 579

Édes, I., Andó, Á., Csanády, M., Mazareán, H., Guba, F. (1983) Cardiovasc. Res. 17, 691

Hoh, J.F.Y., McGrath, P.A., Hale, P.T. (1978) J. Mol. Cell Cardiol. 10, 1053

Hoh, J.F.Y., Yeah, G.P.S., Thomas, M.A.W., Higginbottom, L. (1979) FEBS Lett. 97, 330

Mall, G., Mattfeldt, T., Volk, B. (1980) Virchows Archiv A. Pathol. Anat. Histol. 389, 59

Peterson, G.L. (1977) Anal. Biochem. 83, 346

Porta, E.A., Gomez-Dumm, C.L.A. (1968) Lab. Invest. 4, 352

Regan, T.J., Levinson, G.E., Oldewurtel, H.A., Frank, M.J., Weisse, A.B., Moschos, C.B. (1969) J. Clin. Invest. 48, 397

Schaub, M.C., Tuchschmid, C.R., Srihari, T., Hirzal, H.O. (1984) Muscle and Cell Motility, Abxtract (in press)

Scheuer, J., Malhotra, A., Hirsch, C., Capasso, J., Schaible, T.F. (1982) J. Clin. Invest. 70, 1300

Schreiber, S.S., Briden, K., Oratz, M., Rotschild, M.A. (1972) J. Clin. Invest. 51, 2820

Schwartz, K., Lecarpentier, Y., Martin, J.L., Lompre, A.M., Mercadier, J.J., Swynghedauw, B. (1981) J. Molec. Cell Cardiol. 13, 1071

Schwartz, K., Lompre, A.M., Bouveret, P., Wisnewsky, C., Whalen, R.G. (1982) J. Biol. Chem. 257, 14412

Spodick, D.H., Pigott, V.H., Chirife, R. (1972) N. Engl. J. Med. 287, 677

Takács, Ö., Mészáros, M., Guba, F. (1974) Symposium on the Muscle, Symposia Biologica Hungarica, Akadémiai Kiadó, Budapest

Takács, Ö., Sohár, I., Pelle, T., Guba, F. (1977) Acta Biol. Acad. Sci. Hung. 28, 213

Takács, Ö., Szöőr, Á., Sohár, I., Kesztyűs, L., Guba, F. (1981) Acta Biol. Acad. Sci. Hung. 32, 33

Talbot, D.N., Yphantis, D.A. (1971) Anal. Biochem. 44, 246

Wendt, V.E., Wu, C., Balcon, R., Doty, G., Bing, R.J. (1965) Am. J. Cardiol. 15, 175

Wiegand, V., Stroh, E., Henniges, A., Lossnitzer, K., Kreuzer, H. (1983) Basic Res. Cardiol. 78, 665

Acta Biochim. Biophys. Hung. 21(3), pp. 215-224 (1986)

DYE-LIGAND AFFINITY CHROMATOGRAPHY OF RNA POLYMERASE II

Ivan G. Skripal[x], John R. Weeks[§] and Arno L. Greenleaf[§]

[x]Zabolotney Institute of Microbiology and Virology, Ukranian Academy of Science, Kiev-143, 252143, USSR and
[§]Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA

(Received September 3, 1984)

SUMMARY

The binding of wheat germ RNA polymerase II to five different dye-ligand chromatography gels (Matrex gels, Amicon Corp.) was tested. A quantitative binding of the enzyme to four of the gels, namely Dyematrex gels Blue A, Blue B, Red A and Green A was observed. Only the Orange A gel column failed to bind the enzyme strongly. Nearly 100% of the activity could be recovered from the Green A column by elution with high salt concentration and high pH. Under these conditions only a part of the activity was eluted from the other three columns since the enzyme bound tightly. Enzyme activity could be removed from the columns by elution with nucleotide substrates, but the yield from the Blue A, Blue B and Red A columns was still low (7 to 42%). The Green A Matrex gel appeared to be useful for the purification and analysis of RNA polymerase.

INTRODUCTION

The affinity of RNAp (nucleoside triphosphate: RNA nucleotidyl transferase; EC 2.7.7.6.) to DNA can be exploited for purification procedures by applying it in "affinity" chromatography steps. For this purpose columns of matrix-bound DNA or other matrix-bound substances resembling DNA, such as

Abbreviations: RNAp, DNA-dependent RNA-polymerase; TED, buffer contained 0.05 M Tris-HCl, 0.1 mM EDTA and 1 mM dithiothreitol; TEDG, TED buffer contained 25% (v/v) ethylene glycol; HS, heparin-Sepharose; SDSSG, sodium dodecylsulfate slab gel

Akadémiai Kiadó, Budapest

phosphocellulose or HS (Burgess, 1976), are used. With these materials a high degree of purity of the enzyme isolated from relatively crude mixtures can be achieved. RNAp also binds mononucleotides as substrates thus a chromatography in a medium containing nucleotides may be another useful type for preparing columns to purify and analyse RNAp.

Recently certain dyes covalently linked to a gel matrix have been found to be efficient for purifying enzymes containing nucleotide binding sites, thus the application of "dye-ligand" affinity chromatography is rapidly increasing (Amicon Corp., 1980). In accordance with our findings, Kumar and Krakow (1977) reported that bacterial RNAp is readily bound to Cibacron Blue 3GA, the first dye used widely in dye-ligand chromatography. The subunits of bacterial and yeast RNAp can also be purified on a Cibacron Blue column in the presence of urea (Halling et al., 1977). Binding of wheat germ RNAp II to Cibacron Blue 3GA was also reported by Kumar and Krakow (1977).

In addition, several other dyes linked to gel chromatography materials are now commercially available. We felt that these materials may potentially be useful for studying eukariotic RNAp II. Therefore we have tested the binding ability and recovery of wheat germ RNAp II with five different dye-ligand chromatography substances: Matrex gels Blue A, Blue B, Red A, Orange A and Green A.

MATERIALS AND METHODS

Raw wheat germ was obtained from Dixee Portland Flour Mills (Chattanooga, TN), and was stored at 4°C. All reagents were of analytical or the highest commercially available grade. Matrex gels Blue A, Blue B, Red A, Orange A and Green A (Dyematrex Kit) were obtained from Amicon Corporation.

Buffers were prepared according to Jendrisak and Burgess (1975) and were used in the following compositions (pH values refer to those measured at 5°C): Buffer A: TEDG, pH 7.5 + 0.025 M ammonium sulfate; Buffer B: TEDG, pH 7.5 + 0.25 ammonium sulfate; Buffer C: TEDG, pH 7.5 + 0.5 M ammonium sulfate; Buffer D: TEDG, pH 7.5 + 1.0 M ammonium sulfate; Buffer E: TEDG, pH 8.0 + 0.25 M ammonium sulfate; Buffer F: TEDG, pH 9.0 + 0.05 M ammonium sulfate; Buffer G: TEDG, pH 9.0 + 0.5 M ammonium sulfate.

Buffers for elution of nucleotides were composed of TED, 25% (v/v) glycerol, 0.5 M ammonium sulfate, 10 mM $MnCl_2$, 10 mM $MgCl_2$ and nucleotides as indicated. All buffers used for enzyme purification on heparine-Sepharose also contained 1.5 mM phenylmethanesulfonyl fluoride in a solution of 10 mg/ml dissolved in 95% ethanol together with dithiothreitol, immediately before use.

RNA-polymerase assay. The standard conditions as described by Greenleaf and Bautz (1975) were followed, but thioglycerol was omitted. 10 µg denatured calf thymus DNA (Worthington, Freehold, NY) served as template. Reaction mixtures were incubated for 25 min at 25°C. One unit of activity results in the incorporation of 1 nmole UMP into acid-precipitable material during the incubation period under our assay conditions. The specific radio-activity of $^3|H|$ UTP in the assay mixture was 80 mCi/mmole. One unit of enzyme activity equaled approximately 6.9×10^4 cpm.

HS column. 60 ml of Sepharose 4B (Pharmacia) was activated by CNBr according to March et al. (1974) then 200 mg heparin (sodium salt, grade I, Sigma) was added and incubated overnight at 4°C in 0.1 M $NaHCO_3$ and 0.5 M NaCl. After thorough washing, the excess of reactive compound was removed with 0.1 M ethanolamine (pH 8.0) by incubating for 2 h at room temperature. It was washed thoroughly by alternating the pH between 4 and 9 in 1 M NaCl. HS was stored in 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA. 0.2% (v/v) butanol at 4°C. Before use, the HS was equilibrated with 10 volumes of the appropriate TEDG buffer.

Dyematrex columns. Columns of 2 ml bed volume were treated at 25°C with 20 volumes of 8 M urea and were then equilibrated with 10 ml buffer A at 25°C then again with 10 ml of the same buffer at 4°C.

SDSSG electrophoresis was carried out as described previously (Greenleaf and Bautz, 1975). Proteins were stained with Coomassie Blue.

Glycerol gradient velocity sedimentation. 16 ml linear gradient of 30 to 60% (v/v) glycerol concentration in TED and 0.15 M ammonium sulfate was prepared in a 17 ml polyallomer tube for Sorvall vertical rotor (TV865B). Aliquots of HS enzyme were subjected to sedimentation through the gradient at 60 000 rpm for 12 h at 2°C. Fractions of 0.75 ml were pumped from the bottom of the tube and 5 µl samples were assayed for enzyme activity. The symmetrical enzyme activity peak (see Greenleaf et al., 1979) travelled approximately 2/3 of the distance through the gradient. The recovery was 82%.

Protein and salt assay. Protein concentrations were determined by the method of Bradford (1976). Ammonium sulfate concentrations were determined by conductivity measurements.

RESULTS

RNAp II from 125 g raw wheat germ was partially purified according to Jendrisak and Burgess (1975) on DEAE-cellulose column at 0-4°C. A linear salt gradient eluted the RNAp II

activity (sensitive to 1 µg/ml α-amanitin) in a peak centered at about 0.25 M ammonium sulfate concentration. The fractions containing the activity were pooled and frozen at -80°C (DEAE enzyme: 45 ml, total activity 68 x 10^6 cpm).

Heparin-Sepharose Chromatography

The enzyme obtained by DEAE-cellulose chromatography was further purified on a 5 ml HS column. The DEAE-enzyme was diluted with TEDG buffer to a final ammonium sulfate concentration of 0.05 M. A flow rate of 35 ml/h was applied to the HS column. The enzyme was then eluted with a linear ammonium sulfate gradient of 0.05 to 1.0 M concentration and the fractions were assayed for RNAp activity. As shown in Figure 1, the bulk of the activity was eluted at around 0.55 M salt concentration in a symmetrical peak, while a very small additional activity, about 7% of the total, was eluted at about 0.3 M.

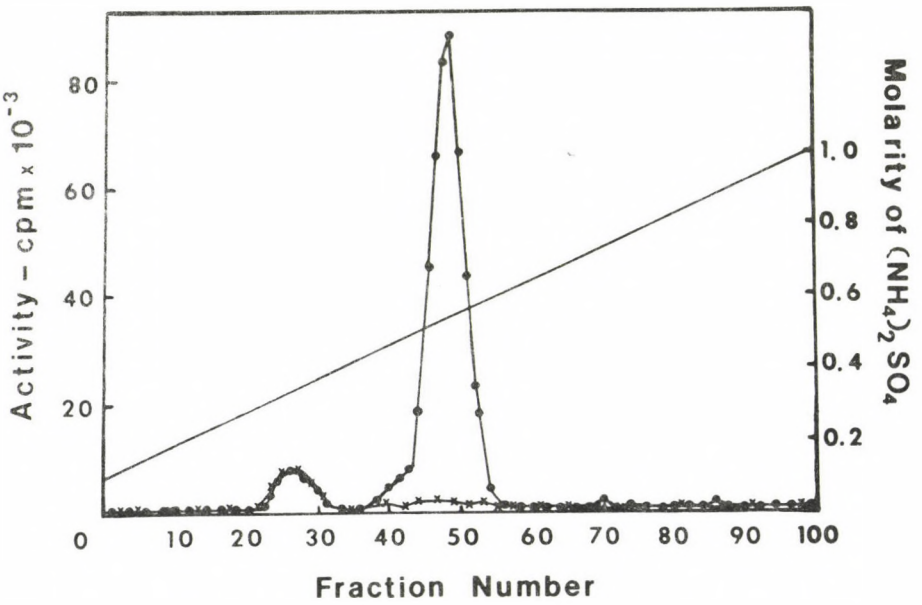


Fig. 1. Heparin-Sepharose chromatography. Material after DEAE-cellulose chromatography was applied to the column and eluted as described in the text. Fractions of 1.5 ml were collected and 20 µl were assayed for RNA polymerase activity in the absence (●) or the presence (x) of 1 µg/ml α-amanitin.

The recovery of activity from the HS column was near to 100%. The major peak appeared to be RNAp II as determined by its sensitivity to α-amanitin. The small peak eluted at lower salt concentration was apparently RNAp I and/or III, since it was insensitive to amanitin. The DEAE column had apparently not separated completely other RNAp-s from RNAp II.

The pure RNAp II fractions were pooled to be used for the studies described below (HS-enzyme: 22.5 ml, total activity 61.3 x 10^6 cpm, 2.9 mg protein). The HS column can be eluted for certain purposes with a salt gradient to obtain more concentrated enzyme solution (Greenleaf et al., 1979).

Th HS-enzyme was not homogeneous, but it was relatively pure, purified approximately 4000-fold, compared to the crude extract and was suitable for a variety of analytical studies. We have used this enzyme preparation to study the binding of RNAp II to a set of dye-ligand affinity column.

Dye Ligand Chromatography

The HS-enzyme was dialyzed against TEDG to 0.025 M ammonium sulfate concentration. To each columm 15 ml enzyme solution (6.2 x 10^6 cpm) was applied and was eluted at 5 ml/h flow rate. The columns were then washed stepwise with discrete volumes of buffer. The flow-through from each volume was collected as separate fractions. After loading with 15 ml sample and washing with 18 ml buffer A, the columns were eluted first with increasing salt gradient of 0.25 M, 0.5 M and 1.0 M ammonium sulfate, pH 7.5 (buffers B, C and D, respectively), then with solutions of increasing pH from 8.0 (buffer E) to 9.0 (buffers F and G), respectively. Each elution step consisted of 2.5 ml buffer. Fractions of 0.5 ml were collected, and 20 µl samples were assayed for activity under conditions described.

All dye ligand columns have bound practically 100% of the applied enzyme activity, except Orange A, to which only about 10% of the activity could be attached (Table 1). The bound enzyme was recovered with varying efficacy from the columns by increasing the salt concentration and then by increasing pH.

In most of the cases very little activity was recovered with either high salt concentration or increased pH (Table 1).

Table 1. Percent recovery of RNA polymerase activity from Matrex gel columns by salt, pH and ligand elution

Column	Flow-through in void volume	Elution[a] by				
		salt	pH	Total recovery	ATP	GTP
Blue A	0	7.2	2.8	10	15	17
Blue B	0	9	3	12	27	42
Red A	0	9.2	0.8	10	7.6	21
Orange A	90	0	10	10	-	-
Green A	0	70	26	96	-	-

[a] elution conditions described in text
- not determined

In contrast, the RNAp II was consistently recovered almost completely from the Green A column: 70% was eluted with high salt concentration and 26% with elevating the pH (Fig. 2). We have eluted the Green A column also with a linear salt gradient of 0.25 M to 2.0 M ammonium sulfate. It was observed that only one salt-eluted peak and the pH-eluted peak persisted (not shown). In addition the 10% of activity that did bind to the Orange A column could be eluted in the "pH peak".

These experiments indicated that RNAp II binds very tightly to four of the Matrex gel columns. Significant activity could be recovered only with Green A. In a separate set of experiments we attempted a more specific elution by using nucleotide substrates to remove the enzyme from the Blue A, Blue B and Red A columns. Enzyme (2.1×10^6 cpm) was applied to each column as described above. A pyrimidine nucleoside triphosphate (CTP) was ineffective in eluting activity from the columns at 0.2 to 15 mM concentrations. On the other hand, 15 mM ATP and GTP in the presence of 0.5 M ammonium sulfate were partially effective (Table 1). The nucleotides were more effective in eluting the enzyme activity than increased salt

concentration or high pH, but recovery (ranging from 7.6 to 42%) never exceeded 50% of the applied activity. The 10% activity that bound to Orange A (Table 1) could be eluted either with ATP or GTP.

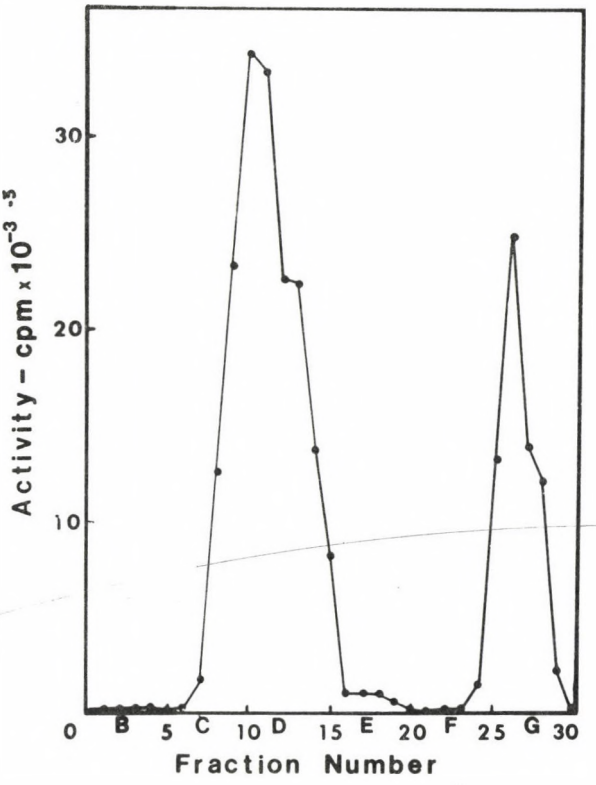


Fig. 2. Dyematrex gel Green A chromatography. Heparin-Sepharose enzyme was dialyzed, applied and eluted with different buffers as follows: fraction 1, Buffer B; fraction 6, Buffer C; fraction 11, Buffer D; fraction 16, Buffer E; fraction 21, Buffer F; fraction 26, Buffer G. 20 µl aliquots of each ffaction were assayed under standard conditions.

Gelelectrophoresis

Since the Green A column appeared to be the most useful for RNAp II purification, we analyzed the composition of the salt and the pH-eluted enzyme preparations by SDSSG electrophoresis. As shown in Fig. 3, these RNAp preparations were

highly pure consisting primarily of polymerase-associated polypeptides (putative subunits, Jendrisak and Burgess, 1977). The enzyme isolated at high pH contained slightly less contaminants. For comparison, a sample of HS enzyme and glycerol-gradient purified enzyme were analyzed simultaneously.

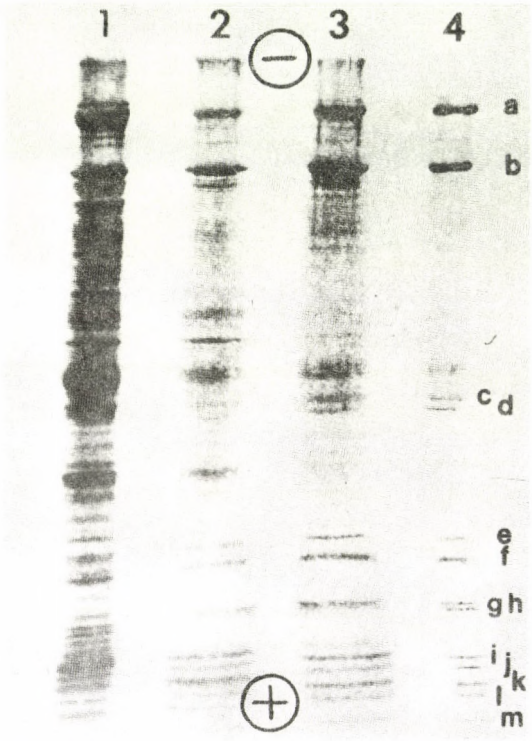


Fig. 3. Polypeptide composition of purified enzymes. The samples were analyzed on a linear acrylamide gradient (7.5-15%) slab gel containing sodium-dodecylsulphate.
Lane 1: heparin-Sepharose enzyme; lane 2: salt-eluted enzyme from Matrex Green A; lane 3: pH-eluted enzyme from Matrex Green A ; lane 4: glycerol-gradient enzyme. Letters indicate putative polymerase II subunits (Jendrisak and Burgess, 1977).

DISCUSSION

Since subunit structure and physical properties of RNAp II from wheat germ are very similar to those of other eukaryotic RNAp IIs (Roeder, 1976), it can serve as a good model for establishing purification procedures applicable to other RNAps.

In fact, we prepared the enzyme by modifying the method of Jendrisak and Burgess (1975). However, instead of a phosphocellulose chromatography we employed a HS column, which was used for purifying RNAp II from Drosophila melanogaster (Greenleaf et al., 1979; Coulter and Greenleaf, 1982). We have found that the yields from HS are almost invariably higher and the column can be run faster as compared to phosphocellulose.

The binding of the HS enzyme to the different dye-ligand chromatography gels was investigated, since some of these are used to purify enzymes that interact with nucleotide substrates, cofactors or effectors. The template and substrate binding properties of RNAp suggested that it may bind tightly also to some of these materials. If so, they might be useful for purifying RNAp.

Of the five Matrex gel dye-ligand columns tested the wheat germ RNAp II was bound tightly to four; to Blue A, Blue B, Red A and Green A. It had little affinity to the Orange A under our conditions. Activity was efficiently recovered from the Green A column by elution with high salt concentration or by pH shift. On the other hand, only 10% of the activity could be eluted from the other three columns. Elution with nucleotide ligands resulted in somewhat higher yields, but not high enough to use the columns for enzyme purification. We could recover inactive enzyme subunits by elution with 8 M urea.

To decide whether the "pH peak" from the Green A column was not simply part of the "salt peak" (see Fig. 2) we have eluted the enzyme from the Green A column with a 0.25 M to 2.0 M ammonium sulfate gradient. A single peak of activity centered at about 0.7 M was eluted and a subsequent elution by high pH (buffer G) still yielded a "pH peak" of activity similar to that shown in Fig. 2.

Thus the Green A column separated the RNAp II into two subfractions. A reasonable explanation for this is that the polymerase can bind it more than one way to the dye-ligand column. Alternatively, the RNAp in the two peaks may differ physically. Differences in the SDS gel patterns of the two en-

zymes can be seen in Fig. 3. The Matrex Green A column appeared to be useful for purification of eukaryotic RNAp II, due to the high binding capacity of the dye-ligand column.

Acknowledgements

This work was supported by NIH grant GM 28078 to Arno L. Greenleaf. We thank D. Coulter for help and advice.

REFERENCES

Amicon Corporation "Dye-Lingand Chromatography" (1980) Lexington, Mass. Publication 512 A

Bradford, M. (1976) Anal. Biochem. 72, 248-254

Burgess, R.R. (1976) R. Losick and M. Chamberlin (eds.) "RNA Polymerase", Cold Spring Harbor Laboratory, New York, pp. 69-100

Coulter, D.E., Greenleaf, A.L. (1982) J. Biol. Chem. 257, 1945-1952

Greenleaf, A.L., Bautz, E.K.F. (1975) Eur. J. Biochem. 60, 169-179

Greenleaf, A.L., Borsett, L.M., Jiamachello, P.F., Coulter, D.E. (1979) Cell 18, 613-622

Halling, S.M., Sanchez-Anzaldo, F.J., Fukuda, R., Doi, R., Meares, C.F. (1977) Fed. Proc. 36, 883

Jendrisak, J.J., Burgess, R.R. (1975) Biochemistry 14, 4639-4645

Jendrisak, J.J., Burgess, R.R. (1977) Biochemistry 16, 1959-1964

Kumar, S.A., Krakow, J.S. (1977) J. Biol. Chem. 252, 5724-5728

March, S.C., Parikh, I., Cuatrecasas, P. (1974) Anal. Biochem. 60, 149-152

Roeder, R.G. (1976) In "RNA Polymerase", R. Losick and M. Chamberlin (eds.), Cold Spring Harbor Laboratory, New York, pp. 285-329

Acta Biochim. Biophys. Hung. 21(3), pp. 225-228 (1986)

ENZYMATIC DETERMINATION OF ISOCITRATE BY AMPEROMETRIC MONITORING OF THE RATE OF OXYGEN CONSUMPTION

Magdolna Ábrahám[*], D. Kirstein[**], F. Scheller[**] and L. Boross[*]

[*]Department of Biochemistry, Attila József University
P.O.Box 533, Szeged, H-6701 Hungary
[**]Academy of Sciences of the GDR, Central Institute of
Molecular Biology, Department of Applied Enzymology,
DDR, Berlin-Buch

(Received April 16, 1985)

SUMMARY

A coupled enzymatic method elaborated for NAD^+-dependent dehydrogenases has been adapted for $NADP^+$-dependent isocitrate dehydrogenase, in combination with amperometric measurements. The isocitrate dehydrogenase activity dependent linearly on the isocitrate concentration in the range 0-2 x 10^{-4} M. Application of this method affords a sensitive estimation of isocitrate even in turbid liquids such as fermentation broths.

Isocitrate is an intermediate in the tricarboxylate cycle. Accompanying citrate fermentation, its level is about a quarter of that of citrate produced by Aspergillus niger (Berry et al., 1977). The level of isocitrate can be determined spectrophotometrically with isocitrate dehydrogenase (Ochoa, 1955; Plaut, 1962).

A new coupled enzymatic method has recently been proposed for the determination of some substrates by dehydrogenases. Cheng and Christian (1977, 1978, 1979) reported that lactate or alcohol was oxidized by NAD^+ coenzyme in the presence of the appropriate dehydrogenase. Finally, NAD^+ was regenerated by molecular oxygen, in a redox reaction catalysed by horseradish peroxidase in the presence of Mn^{2+} ions and certain

Abbreviations: IDH, isocitrate dehydrogenase; HRP, horse radish peroxidase; DCP, dichlorophenol

Akadémiai Kiadó, Budapest

phenols as cocatalysts. The oxygen depletion was proportional to the amount of substrate in the solutions and was monitored by an amperometric oxygen electrode connected to a glucose analyser.

Here we report a coupled enzymatic method for the measurement of isocitrate, using NADP-linked IDH and HRP according to the following reaction:

$$\text{isocitrate} + \text{NADP}^+ \xrightleftharpoons[Mn^{2+}]{\text{IDH}} \alpha\text{-ketoglutarate} + CO_2 + \text{NADPH} + H^+$$

$$\text{NADPH} + H^+ + 1/2\ O_2 \xrightarrow[Mn^{2+}\ \text{DCP}]{\text{HRP}} \text{NADP}^+ + H_2O$$

The isocitrate concentration was determined in a reaction mixture containing 0.07 U/ml IDH, 0.5 U/ml HRP, 1.6×10^{-4} M $NADP^+$, 10^{-4} M $NADP^+$, 10^{-4} M DCP and 1.3×10^{-4} M $MnCl_2 \times 4H_2O$ in 0.1 M Tris-succinate buffer, pH 8.0. The total volume of the mixture was 5 ml. The final concentration of isocitrate was varied up to about 10^{-4} M (Fig. 1).

In a thermostated cell the rate of oxygen consumption was determined with Clark-type oxygen electrodes (Radelkis, Budapest, Hungary and Metra, Radebeul, GDR). These electrodes were combined either with a glucometer (GKM 01, Scientific Instruments Centre, Acad. Sci. GDR), for derivative measurements (dI/dt), or with a Radelkis amperometric glucose adapter, connected to a potentiometric recorder (OH-814/1/Radelkis, Budapest, Hungary).

The glucometer was calibrated in μA/min using a polarograph (GWP 673, Acad. Sci. GDR). The Radelkis instrument was used to measure stationary currents, mV readouts on the recording mV-meter. All measurements were performed at 298 K.

When glucometer was used the extent of the rapid decrease of the voltage (mV) was proportional to the concentration of the isocitrate in a concentration range of 0 to 2.0×10^{-4} M (Fig. 1).

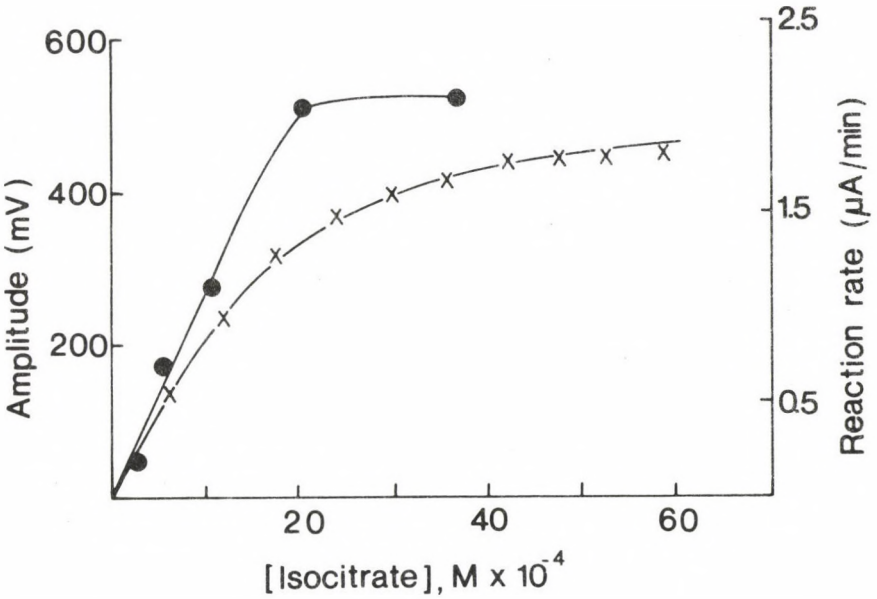


Fig. 1. Calibration graph for isocitrate standard, measured with an amperometric glucose adapter connected to a mV-meter (●) and a glucometer (x).

In the case of measurements with amperometric glucose adapter the rate of decrease of the current (µA/min) depended characteristically on the concentration of isocitrate. A linear relationship was found up to about 1.5×10^{-4} M. The response showed a saturation curve-type function on the concentration (Fig. 1).

The optimum concentrations of NADP, IDH and HRP for the measurements were determined to be $1.5\text{-}2.0 \times 10^{-4}$ M, 0.07-0.1 V/ml and 0.5-0.63 V/ml, respectively.

According to our results the amperometric monitoring of oxygen consumption in the $NADP^+$-containing HRP/cocatalyst mixture was successfully applied for a sensitive determination of isocitrate concentration. Use of the amperometric method in industrial or laboratory practice can afford a rapid possibility for the estimation of isocitrate also in turbid solutions as fermentation broths.

REFERENCES

Berry, D.R., Chmiel, A., Al.Obaidi, Z. (1977) In: Genetics and Physiology of Aspergillus p. 39, Academic Press, New York

Cheng, F.S., Christian, G.D. (1977) Anal. Chem 49, 1785-1788

Cheng, F.S., Christian, G.D. (1978) Clin. Chem. 24, 621-626

Cheng, F.S., Christian, G.D. (1979) Clin. Chem. Acta 91, 295-301

Ochoa, S. (1955) In: Methods in Enzymology (Colowick, S.P., Kaplan, N.O. eds.) Vol. 1, p. 699, Academic Press, New York

Plaut, G.W.E. (1962) In: Methods in Enzymology (Colowick, S.P., Kaplan, N.O. eds.) Vol. 5, p. 645, Academic Press, New York

Acta Biochim. Biophys. Hung. 21(3), pp. 229-236 (1986)

METABOLISM OF NON-ENZYMIC GLYCOSYLATED LOW DENSITY LIPOPROTEIN BY MINI PIG AORTIC ENDOTHELIAL CELLS

Pál I. Bauer, Kálmán G. Büki, Éva Csonka*, Sándor A. Koch* and István Horváth

Second Institute of Biochemistry and Second Institute of Pathology*, Semmelweis University Medical School, H-1444 Budapest, Box 262, Hungary

(Received February 20, 1986)

SUMMARY

[U-^{14}C]-glucose was incorporated non-enzymatically into the protein moiety of human low density lipoprotein. The incorporation was time and glucose concentration dependent. Investigated mini pig aortic endothelial cells showed that the glycosylated low density lipoprotein was bound, internalized and degraded significantly less than that of the native one measured with [^{125}-I]-low density lipoprotein.

INTRODUCTION

There is a large amount of rapidly accumulating data available in the literature about the non-enzymic glycosylation of plasma proteins. The minor hemoglobin (Hb_{A-1c}) was the first example to show that glucose can react with the amino terminal of β-chain (1). Recently, glycosylation was reported of eye lens crystalline (2), erythrocyte membrane proteins (3, 4), serum albumin (5, 6), fibrinogen (7) and lipoproteins (8-10). The amount of glucose bound to these proteins is enhanced in diabetes (1, 3-5, 6, 8). Schleicher et al. (8) showed that glucose was bound to the ε-NH_2 moiety of lysine(s) in low density lipoprotein (LDL).

Abbreviations: LDL, low density lipoprotein; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate bufferred saline; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

Akadémiai Kiadó, Budapest

The chemical modification of the lysine residues, i.e. methylation (11), acetylation (12), malondialdehyde treatment (13), cationization (14) in the LDL molecule, can alter the interaction of LDL molecule with its specific receptors present in various cell types (11-13). When LDL was cationized (N,N-dimethyl-1,3-diaminopropane was coupled to the ε-NH_2 groups of lysines in the LDL) the modified lipoprotein was taken up by the cells via the nonspecific receptor pathway resulting in the increase of cellular cholesterol-ester content and the decrease of the endogenous cholesterol synthesis (15). The enhanced cellular cholesterol content may cause lipid deposition in the arterial wall. Because glycolysation makes the NH_2 groups of the protein more basic, we investigated the binding, internalization and degradation of glycosylated LDL by cultured mini pig aortig endothelial cells.

MATERIALS AND METHODS

Dulbecco's modified Eagle medium, fetal calf serum were obtained from Gibco and Seromed, respectively. All other reagents were the products of Reanal Fine Chemicals (Budapest). [^{125}-I]-NaI (carrier free) was the product of the Isotopic Institute of the Humgarian Academy of Sciences. [U-^{14}C]-Glucose was purchased from Amersham.

Human LDL was isolated from the plasma of healthy donors by sequential ultracentrifugation at densities between 1.019 and 1.063 (16). LDL was dialysed against PBS containing 0.2 mM EDTA overnight, then filtered through a membrane filter (0.45 µm, pore size). LDL was glycosylated by incubation of 0.4 ml of LDL (10 mg protein/ml) in PBS with the indicated amount of glucose (Figs. 1, 2) in the presence of 0.02% Na-azide and 10 mM Hepes, pH 7.4, at 37°C, under sterile conditions. The [U-^{14}C]-glucose may contain some unidentified material, which react rapidly with proteins and invalidated the results of *in vitro* labelling experiments (17). Therefore, it was pretreated with serum albumin (1 mg/ml in PBS) for 48 h at 4°C. The serum albumin was removed by gelfiltration through a Sephadex G25 column equilibrated with PBS. Samples containing only free glucose were pooled and sterilized. The LDL was radioiodinated via the iodine-monochloride procedure (18). Unbound ^{125}I was removed by gelfiltration on Sephadex G200 followed by exhaustive dialyses against 3000 fold volume of PBS containing 0.2 mM EDTA. The specific activity varied between 320 and 700 cpm/ng protein. Mini pig aortic endothelial cells were cultivated as described before (19). The cells were identified as endothelial by the following criteria: (i) morphological appearence (20) and (ii) presence of the angiotensin converting enzyme activity activated

by Co^{2+}, and inhibited by SQ 20881 as described by Hayes et al. (21). For the experiments, cells were grown in scintillation vials in DMEM supplemented with 20% fetal calf serum at 37°C, in a humidified incubator in 5% CO_2 atmosphere. Subconfluent cultures were used. The cells were washed *in situ* twice with prewarmed PBS containing 2.5 mg/ml human serum albumin. As incubation medium we used 0.5 ml of $|^{125}I|$-LDL containing DMEM, supplemented with 10 mM Hepes, pH 7.4 and with 2.5 mg/ml albumin. Binding was measured at 4°C for 6 or 18 hrs (22). At the end of incubation, the cells were washed with 5 x 2 ml PBS containing 0.2% albumin and once with PBS only. Four ml of scintillation cocktail, toluene base scintillation cocktail-Triton X-100, 2:1 (v/v) was added and the radioactivity of the samples were determined in a Beckman LS 350 liquid scintillation spectrometer. The binding and uptake was differentiated by incubating the cells with 10 mg/ml of heparin after the washing process as described by Goldstein et al. (23). Degradation was measured as described by Bierman et al. (24). In brief, after incubation 0.4 ml of the medium was withdrawn and deproteinized with a final concentration of 10% TCA. The acid soluble fraction was counted to determine the content of degradation products after treatment with hydrogen peroxide and extraction of free iodine with chloroform. To determine the spontaneous breakdown, empty vial controls were used. Protein was determined with the method of Lowry et al. (25) using bovine serum albumin as standard.

RESULTS

Under the experimental conditions used $[U\text{-}^{14}C]$-glucose was incorporated into the protein part of human LDL. The time dependence of incorporation shows a saturation kinetics, while the incorporation of $[U\text{-}^{14}C]$-glucose into the LDL is nearly linear with glucose concentrations up to 55 mM (Figs. 1 and 2). When glucose concentration was 5.5 mM approx. 2-3 moles of glucose was incorporated per mole of apoprotein B with an assumed mol. weight of 250 000. The incubation mixture contained neither $NaBH_4$ nor $NaCNBH_3$, thus we suppose that incorporation was in Schiff's base form or in ketoamine form after the Amadori rearrangement.

We studied the metabolization of LDL by mini pig aortic endothelial cells using native and glycosylated LDL. The binding studies showed that glucose pretreatment of LDL caused a marked decrease in the binding of human LDL to endothelial cells. The inhibition reached approx. 70% when LDL was pretreated with 55 mM glucose for 72 h, at 37°C (Fig. 3).

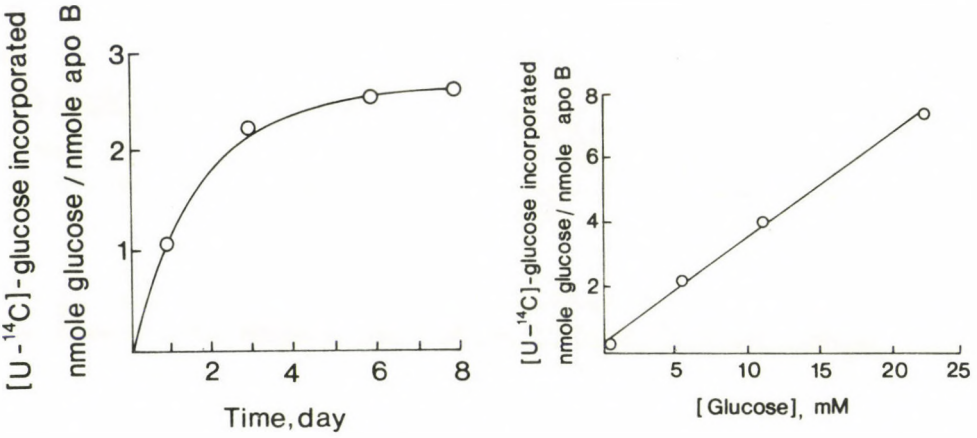


Fig. 1. (left) Time dependent incorporation of [U-^{14}C]-glucose into LDL. In a total volume of 1 ml, 10 mg LDL, 5.5 µmole of [U-^{14}C]-glucose (10 µCi/5.5 µmole) 10 µmole of Hepes, pH 7.4, 10 µmole of Na-phosphate, pH 7.4 and 140 µmole of NaCl were incubated at 37°C. At times indicated, 200 µl of samples were withdrawn and protein-bound radioactivity was determined as described in Materials and Methods.

Fig. 2. (right) Incorporation of [U-^{14}C]-glucose into LDL as a function of glucose concentration. In a total volume of 0.2 ml there were: [U-^{14}C]-glucose (10 µCi/mg) as indicated, 2 mg of human LDL, 10 µmole of Hepes, pH 7.4, 10 µmole of Na-phosphate, pH 7.4, 140 µmole of NaCl and 0.2 mg of NaN_3. Incubation was for 72 hrs at 37°C, then samples were taken and protein-bound radioactivity determined as described in Materials and Methods.

The same extent of inhibition was observed when the incubation time was 18 h or when 5 mg/ml [^{125}I]-LDL concentration (hyperlipidemic condition) was used (data not shown). At 37°C inhibition of binding and uptake was also observed. Fifty percent inhibition was found with LDL pretreated with 55 mM glucose for 72 h, at 37°C (Fig. 3B). Inhibition was also observed when the bound LDL was dissociated from the cells by heparin treatment at 37°C (data not shown).

The LDL taken up by the specific or by the scavenger pathways is hydrolysed in the lysosomal system of the cell. Mono-

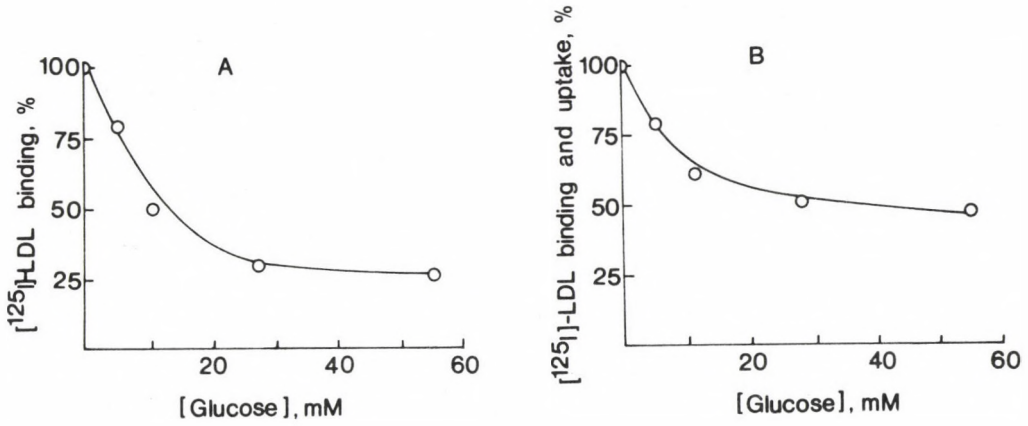


Fig. 3. Effect of glucose pretreatment on the binding and uptake of human LDL to mini pig aortic endothelial cells. Pretreatment of human LDL with glucose was in a total volume of 0.2 ml containing 2 mg of LDL, glucose as indicated on the abscissa, in PBS, supplemented with 10 mM Hepes, pH 7.4 and with 0.02% Na-azide. After incubation for 72 hrs at 37°C, samples were gelfiltered on Sephadex G25 columns and the lipoproteins were iodinated as described in Materials and Methods. 2 x 10^5 endothelial cells were incubated with 100 µg/ml [^{125}I]-LDL for 4 hrs at 4°C (A).

After incubation cell bound radiocativity was determined as described in Materials and Methods. Fig. 3B shows the binding and uptake of LDL by endothelial cells as a function of glucose concentration. The experiment was made as described above, the only difference being that incubation was carried out at 37°C.

or diiodotyrosine is formed and secreted into the incubation medium. We investigated the rate of LDL degradation using native or glycosylated LDL. The data in Table 1 show that glycosylation inhibited also degradation by 50%, apparently due to the limited uptake.

Table 1. Degradation of native and glycosylated LDL by cultured endothelial cells[a]

LDL conc. µg/ml	Degradation: 10% TCA soluble dpm/10^5 cells	
	LDL	glycosylated LDL
100	4754	2272
5000	41330	23965

[a] LDL was pretreated with 55 mM glucose (37°C, 72 h) gelfiltered on Sephadex G25, radioiodinated. 2 x 10^5 endothelial cells were incubated with the indicated amount of [^{125}I]-LDL for 6 hrs, at 37°C. TCA soluble radioactivity in the medium was determined.

DISCUSSION

Human LDL incorporated [U-^{14}C]-glucose into the protein moiety of the molecule. The incorporation was time and concentration dependent. The binding of glucose to LDL is very stable, it does not dissociate during dialysis, gelfiltration nor by TCA precipitation. On incubation at 37°C for 48-72 h, the extent of incorporation was 2-3 moles of glucose per mole of apoprotein B at a glucose concentration of 5.5 mM. This value is the same as described by Schleicher et al. (8). They found 1.9 moles glucose incorporated per mole apoprotein of LDL isolated from persons suffering from diabetes mellitus. When the incubation mixture contains $NaCNBH_3$ as reducing agent, the incorporation goes up to 10 mole glucose per mole apoprotein B as described by Gonen et al. (9).

The cationization process of LDL alters the LDL-cell interaction. The cationized LDL binds first to anionic sites randomly distributed over the cell surface, thus, bypassing the specific receptor route, and it is taken up by the cells with adsorptive endocytosis and enhances the cells' cholesterol content. Similarly this phenomenon was observed with fibroblasts (12), bovine endothelial and smooth muscle cells (15). Glucose modifies the

pK of $-NH_2$ group, i.e. when ketoamine bond is formed the amino group becomes more basic. Gonen (9) and Sasaki (10) found that glucose pretreatment of LDL reduced its metabolization by fibroblast cells relative to native LDL. Despite these results, knowing the very severe consequences of angiopathia in diabetes, it was worth investigating the effect of glycosylation on LDL metabolism of cultured aortic endothelial cells. In our experiments, similarly to those made on fibroblasts (9-10), the modified LDL was bound, degraded and taken up at a significantly lower extent relative to the native LDL up to 5 mg/ml LDL concentration. It seems that the uptake takes place through the same extent with unlabelled native and glycosylated LDL and vice versa (data not shown). How the glycosylation of LDL alters its in vivo metabolisation needs further investigations.

Moreover, glycosylation of lysines in the LDL and its metabolism by endothelial cells may reveal new aspects in the receptor binding moiety of LDL in two ways. Firstly, glucose modifies very specifically the lysines involved in the binding of LDL to its receptor. Only about 2-3 moles of lysines are modified per mole of apoprotein B and this modification causes 70% inhibition of binding. When LDL is modified by acetylation (12), methylation (11) or by malondialdehyde treatment (13) all of the 126 lysine residues in the apoprotein B are modified. Secondly, the lysine(s) involved in the binding to the receptor has to be in an environment in the LDL molecule, where the $-NH_2$ group of the lysine involved in the binding process is deprotonated at pH 7.4, because free $-NH_2$ group is obligatory in the Schiff base formation followed by Amadori rearrangement (6).

REFERENCES

1. Holmquist, W.R., Schroeder, W.A. (1966) Biochemistry 5, 2489-2503
2. Stevens, V.J., Rouzer, G.A., Monnier, V.M., Cerami, A. (1978 Proc. Nat. Acad. Sci. USA 75, 2918-2922
3. Bunn, H.F., Briehl, R.W. (1970) J. Clin. Invest. 49, 1088-1095

4. Schleicher, E., Scheller, L., Wieland, O.H. (1981) Biochem. Biophys. Res. Commun. 99, 1011-1019

5. Bailey, A.J., Robins, S.P., Tanner, M.J.A. (1976) Biochim. Biophys. Acta 434, 51-57

6. Dolhofer, R., Wieland, O.H. (1979) FEBS Lett. 103, 282-286

7. McVerry, B.A., Thorpe, S., Joe, F., Gaffney, O., Huehns, E.R. (1981) Haemostasis 10/5, 261-271

8. Schleicher, E., Denfel, T., Wieland, O.H. (1981) FEBS Lett. 129, 1-4

9. Gonen, B., Baenzinger, J., Schonfeld, J., Jacobson, D., Farrar, P. (1981) Diabetes 30, 875-878

10. Sasaki, J., Cottam, G.L. (1982) Biochem. Biophys. Res. Commun. 104, 977-983

11. Weisgraber, K.H., Innerarity, T.L., Mahley, R.W. (1978) J. Biol. Chem. 253, 9053-9062

12. Basu, S.K., Goldstein, J.L., Anderson, R.G.W., Brown, M.S. (1979) Proc. Nat. Acad. Sci. USA 75, 3179-3182

13. Fogelman, A.H., Shechter, I., Seager, J., Hokom, M., Child, J.S., Edwards, O.S. (1980) Proc. Nat. Acad. Sci. USA 77, 2214-2218

14. Danon, D., Goldstein, L., Marikovsky, Y., Skulteisky, E. (1972) J. Ultrastruct. Res. 38, 500-510

15. Fielding, P.E., Vlodavsky, I., Gospodarowicz, D., Fielding, C.J. (1979) J. Biol. Chem. 254, 749-755

16. Havel, R.J., Eder, H.A., Bragdon, J.H. (1955) J. Clin. Invest 34, 1345-1353

17. Trueb, B., Holenstein, C.G., Fischer, R.W., Winterhalter, K.H. (1980) J. Biol. Chem. 255, 6717-6720

18. Bilheimer, D.W., Eisenberg, S., Levy, R.I. (1972) Biochim. Biophys. Acta 260, 212-221

19. Csonka, E., Kerényi, T., Koch, A.S., Jellinek, H. (1975) Arterial Wall 3, 31-37

20. Csonka, E., Szemenyei, K., Miskulin, M., Robert, A.M. (1980) Artery 8, 235-258

21. Hayes, L.W., Gognen, C.A., Ching, S.F., Slakey, L.B. (1978) Biochem. Biophys. Res. Commun. 82, 1147-1153

22. Goldstein, J.L., Brown, M.S. (1974) J. Biol. Chem. 249, 5153-5162

23. Goldstein, J.L., Basu, S.K., Brunschede, G.Y., Brown, M.S. (1976) Cell 7, 85-95

24. Bierman, E.L., Stein, O., Stein, Y. (1974) Circ. Res. 35, 136-150

25. Lowry, O.H., Rosebrough, N.I., Farr, A.L., Randall, L.Y. (1951) J. Biol. Chem. 193, 265-275

Acta Biochim. Biophys. Hung. 21(3), pp. 237-245 (1986)

INVESTIGATION ON THE BINDING OF TRYPTOPHAN ENANTIOMERS TO HUMAN SERUM ALBUMIN

Ilona Fitos, Miklós Simonyi

Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

(Received January 3, 1983)

SUMMARY

The binding of radiolabelled tryptophan enantiomers to human serum albumin was investigated by ultrafiltration and by the microparticle technique. L-Trp was found to exhibit a high degree of secondary binding. D-Trp showed increased degree of binding when the HSA concentration was decreased. Stereoselective binding has also been detected in stereoselectively labelled racemic mixtures. Both L- and D-Trp were found to compete for the primary binding site with specific benzodiazepine markers. All the experiments indicate that stereoselectivity of binding is much lower than generally believed.

INTRODUCTION

Tryptophan (Trp) binding to albumin is the classical example for the manifestation of stereoselectivity in binding to serum proteins. McMenamy and Oncley (1958) in their comprehensive study of Trp binding reported that the binding affinity for the D-enantiomer was 100-fold lower than of L-Trp, and very often this high figure is referred to (Alebic-Kolbah et al., 1979b; Bruderlein and Bernstein, 1979; Kragh-Hansen, 1981) as the degree of stereoselectivity. In fact, this factor was estimated from the Scatchard plots of the enantiomers obtained

Abbreviations: HSA, human serum albumin; OAc, oxazepam acetate; Trp, tryptophan

Correspondence: M. Simonyi
Central Research Institute for Chemistry
Budapest, Pf. 17. H-1525

Akadémiai Kiadó, Budapest

with bovine mercaptoalbumin (i.e. not with HSA) and further by assuming that while one specific binding site exists for L-Trp, the binding of D-Trp is not specific and the number of its binding sites is as large as approximately 25. Thus, the factor of 100 reflects the ratio of slopes of the Scatchard plots obtained for Trp enantiomers with the smaller slope being close to zero. On the other hand, King and Spencer (1970) found D-Trp competitively displacing L-Trp from bovine albumin. In practice, D-Trp was subjected to very few binding studies as a possible consequence of its biological insignificance, the lack of commercial radioactive compound and the experimental difficulties encountered in measuring binding constants of low affinity.

Recently, the stereoselectivity of Trp binding to human serum albumin was studied by chromatography and NMR. While no binding for D-Trp was detected by HPLC (Sebille and Thuaud, 1980), Lagercrantz et al. (1981) determined the strongest association constants for L-Trp and D-Trp by affinity chromatography as being 1.1×10^4 M^{-1} and 1.3×10^3 M^{-1}, respectively, and thus found the binding stereoselectivity to be as low as 8. The NMR results indicated that the binding induced similar changes in the proton spectra of the two stereoisomers and no qualitative difference between them could be observed (Monti et al., 1977).

This work presents direct binding data for both enantiomers. The stereoselectivity of binding was tested in competition experiments, too.

MATERIALS AND METHODS

HSA (fatty acid free, Fraction V.) was purchased from Miles Laboratories. L-, D- and DL-Trp (puriss) were obtained from Fluka AG. [^{3}H]L-Trp (263 GBq/mmol) and [^{14}C]DL-Trp (1.78 GBq/mmol) purchased from NEN were used to label radioinactive Trp solutions. Since the amount of radioactive Trp applied for labelling has always been diminishingly low, Trp concentrations of labelled samples equaled those of the radioinactive compounds. [^{14}C]D-Trp was prepared by resolution of the above racemate on a column containing HSA immobilized on CNBr-activated Sepharose matrix (Lagercrantz et al., 1981).

The entirely separated two peaks represented specific activities of 11.1 MBq/ml and 5.8 MBq/ml for D- and L-enantiomers, respectively. Identical properties of [^{14}C]L-Trp and [^{3}H]L-Trp in binding to HSA were found and taken as evidence for the enantiomeric purity of resolved enantiomers. [^{3}H]Diazepam (185 GBq/mmol) was purchased from the Institute of Isotopes, Budapest. The synthesis of [^{14}C]dl-oxazepam acetate (OAc, 96.2 MBq/mmol) has been described (Tegyey et al., 1979).

Solutions were prepared in Ringer buffer (pH 7.4). Binding studies were carried out at room temperature by ultrafiltration technique using Amicon PM-10 membranes for Trp and YM-10 membranes for OAc. The concentration of HSA was varied form 3.77×10^{-5} M to 3.02×10^{-4} M in ultrafiltration experiments and kept constant in each series performed to determine the Scatchard relation. Since diazepam has considerable binding to ultrafiltration membranes, competition with diazepam binding was measured by the technique using HSA immobilized in polyacrylamide microparticles (Sjöholm et al., 1979). In these experiments 5 mM phosphate buffer with 0.1 M KCl (pH 7.4) and HSA concentration of 1.28×10^{-5} M were applied.

Free fractions ($\alpha = c_{free}/c_{total}$) were measured by liquid scintillation counting of radiolabelled ligands. This technique enabled us to take measurements only on the labelled enantiomers in stereoselectively labelled racemic Trp solutions*.

RESULTS AND DISCUSSION

Binding experiments were carried out by varying the ligand concentration (c_{Trp}) between 10^{-5} and 10^{-2} M. Data are shown in Fig. 1. As apparent, increasing protein concentration is accompanied by impaired binding affinity (cf. curves a, b and c) in agreement with Bowmer and Lindup (1978), Bruderlein and Bernstein (1980) who found similar results for L-Trp binding to bovine serum albumin and to HSA, respectively. As shown in Fig. 1, the same phenomenon holds for D-Trp binding (cf. curves d and e) that has not yet been observed. No difference could be observed between [^{14}C]L-Trp and [^{3}H]L-Trp (cf. curve b in Fig. 1) in binding experiments, which tends to indicate that the resolution of [^{14}C]DL-Trp was perfectly performed (see Materials and methods).

A very striking feature of the Scatchard plots for L-Trp binding is the high contribution of the generally neglected

Radioactive labelling is denoted by.

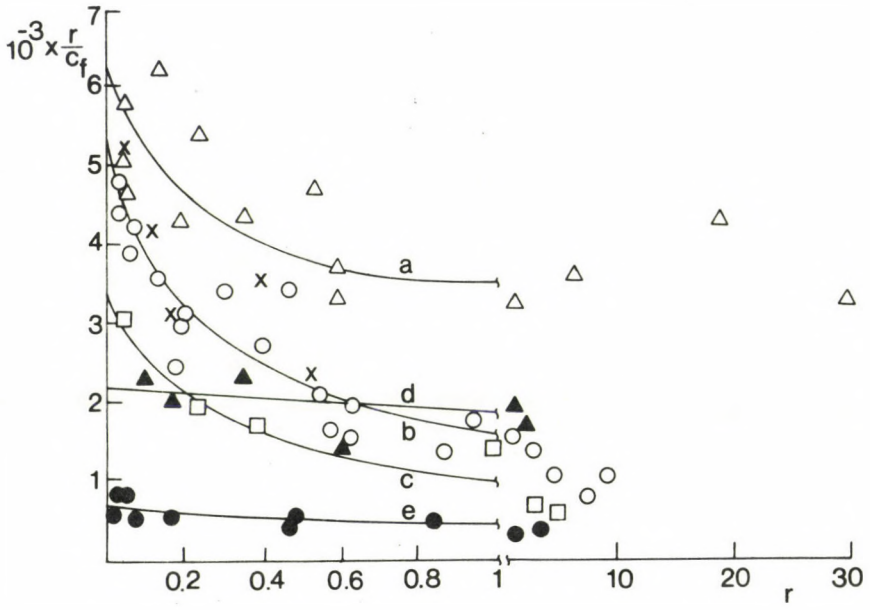


Fig. 1. Scatchard plots for the binding of Trp enantiomers to HSA as determined from ultrafiltration experiments; r equals the moles of Trp bound by one mole of HSA; c_f is the concentration of free Trp.
Curve a and Δ: [^{3}H]L-Trp, $c_{HSA} = 3.77 \times 10^{-5}$ M;
curve b and o: [^{3}H]L-Trp, $c_{HSA} = 1.51 \times 10^{-4}$ M;
x: [^{14}C]L-Trp, $c_{HSA} = 1.51 \times 10^{-4}$ M;
curve c and □: [^{3}H]L-Trp, $c_{HSA} = 3.02 \times 10^{-4}$ M;
curve d and ▲: [^{14}C]D-Trp, $c_{HSA} = 3.77 \times 10^{-5}$ M;
curve e and ●: [^{14}C]D-Trp, $c_{HSA} = 1.51 \times 10^{-4}$ M.

non-saturable secondary binding. Considering the low accuracy of the data it would be hazardous to make extrapolations to the intercept of the horizontal axis by any curve fitting procedure. These curves allow us to compare only the vertical intercepts characterizing the Σnk values (Vallner et al., 1976) and show stereoselective ratios of ∿ 8 (curves b and e) and ∿ 3 (curves a and d) in favour of the L-isomer for 1% and 0.25% HSA concentrations, respectively. It is to be noted that a significant difference exists between the enantiomers even in the region of high saturations, which indicates stereoselectivity in the binding of Trp enantiomers to secondary binding sites, too.

Stereoselectivity was also studied by ultrafiltration of stereoselectively labelled racemic mixtures. The free fractions for L-Trp* in the presence of equimolar amounts of D-Trp* (α_{L^*D}), as well as for DL-Trp* ($\alpha_{L^*D^*}$) were measured and the corresponding α_{LD^*} values were derived. Table 1 shows the α values in racemates and the ratios of enantiomeric binding constants (K_L/K_D) calculated with the assumption that the enantiomers occupy identical binding sites. Though this is an indirect method with low accuracy, stereoselectivity is undoubtedly small.

Table 1. Free fractions (α) and stereoselectivity in racemic mixtures

$10^5 \times c_{HSA}$ (M)	$10^5 \times c_{Trp}$ (M)	α_{L^*D}	$\alpha_{L^*D^*}$	$(\alpha_{LD^*})^+$	$(\frac{K_L}{K_D})^{++}$
15.1	2.30	0.66	0.76	0.86	3.2
	5.33	0.68	0.76	0.84	2.5
	9.33	0.70	0.77	0.84	2.3
	10.15	0.70	0.78	0.86	2.6
	20.35	0.75	0.83	0.91	3.4
	33.70	0.77	0.83	0.89	2.4
3.77	1.02	0.82	0.86	0.90	2.0
	2.81	0.82	0.87	0.92	2.5
	5.33	0.89	0.91	0.93	1.6
	9.33	0.87	0.89	0.91	1.5
	20.35	0.86	0.90	0.94	2.6

$^+ \alpha_{LD^*} = 2\,\alpha_{L^*D^*} - \alpha_{L^*D}$ (Simonyi et al., 1980a)

$$^{++}\frac{K_L}{K_D} = \frac{(1-\alpha_{L^*D})\,\alpha_{LD^*}}{\alpha_{L^*D}\,(1-\alpha_{LD^*})}$$

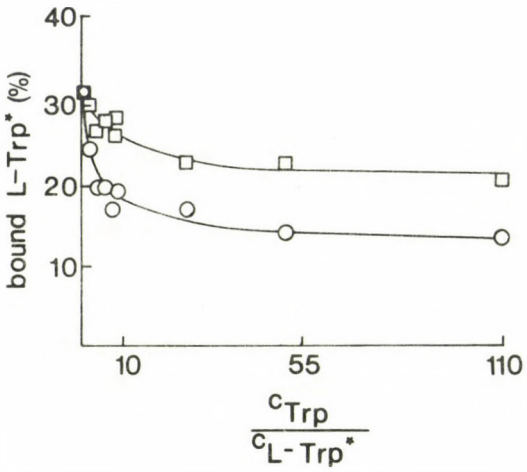


Fig. 2. Influence of L-Trp (o) and D-Trp (□) on the binding of [^{3}H]L-Trp as determined by ultrafiltration;
$c_{L\text{-}Trp^*} = 9.35 \times 10^{-5}$ M; $c_{HSA} = 1.51 \times 10^{-4}$ M.

Two series of displacement experiments were carried out in which the influence of increasing amounts of L-Trp as well as of D-Trp on the binding of L-Trp* was followed. Fig. 2 shows the decrease in the bound fraction upon addition of the unlabelled enantiomers to L-Trp*-containing samples in quantities ranging from the equimolar up to a hundred-fold excess. It can be seen that both Trp enantiomers partially displace the bound L-Trp* marker and that L-Trp is clearly a more effective displacer. The two curves are similarly asymptotic and their difference corresponds to a stereoselectivity of 2 to 6, if competitive inhibition is assumed. Fig. 2 again shows that secondary binding sites are non-saturable even for L-Trp. Hence, the supposition of King and Spencer (1970) on the competitive displacement of L-Trp by F-Trp seems to apply only to the primary binding site on HSA. The fact that L-Trp* also binds to secondary sites makes it a wrong marker of the specific binding site.

The specific binding site for L-Trp on HSA was found to be identical with the benzodiazepine binding site (Sjöholm et al.,

1979; Müller and Wollert, 1975a). More informative displacement experiments were performed with unlabelled Trp enantiomers and labelled benzodiazepines. Fig. 3 shows the effect of L-Trp and D-Trp on the binding of diazepam. While L-Trp is again a more potent displacer than its enantiomer, contrary to Fig. 2, the displacement of [^{3}H]diazepam monotonously increases with increasing concentrations of both Trp enantiomers. Hence, Fig. 3 clearly demonstrates that D-Trp also competes for the specific L-Trp binding site.

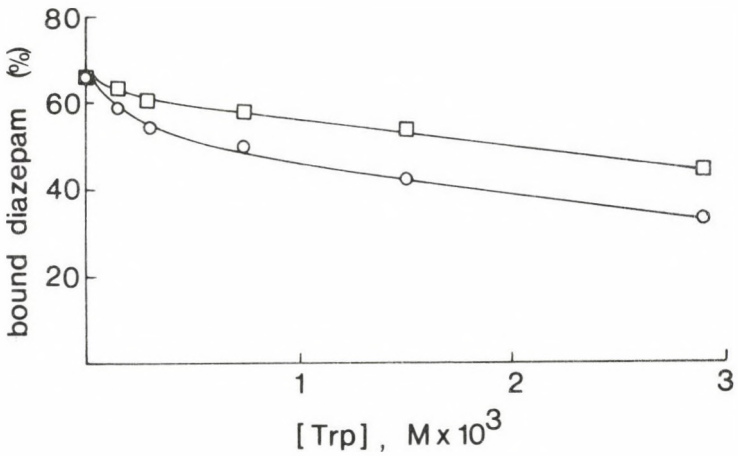


Fig. 3. Competition of L-Trp (o) and D-Trp (□) with the binding of [^{3}H]diazepam to HSA immobilized in microparticles;
$c_{diazepam} = 1.12 \times 10^{-5}$ M;
$c_{HSA} = 1.28 \times 10^{-5}$ M.

In full agreement, with this finding, Table 2 shows the stereoselective increase of the free fraction of OAc$^{\text{R}}$ upon the addition of Trp enantiomers. D-Trp displays again its ability to displace the bound benzodiazepine marker.

When the experiments were repeated with HSA containing natural fatty acids, the results were similar but with even smaller degrees of binding for both stereoisomers.

Summarizing the above experimental evidence, the following conclusions can be drawn: i/ the binding of Trp enantiomers to

HSA is qualitatively similar; ii/ D-Trp does bind to the specific site; iii/ the overall stereoselectivity of binding is smaller than 10.

Table 2. The effect of L-Trp and D-Trp on the free fraction of [^{14}C]dl-oxazepam acetate (OAc^{*})

c_{Trp} (M)	$\alpha_{OAc^{*}}$ (+ L-Trp)	$\alpha_{OAc^{*}}$ (+ D-Trp)
0	0.38	0.38
1.70×10^{-4}	0.41	0.39
9.80×10^{-4}	0.51	0.41
1.22×10^{-2}	0.61	0.45

$c_{OAc^{*}}$: 4.49×10^{-5} M; c_{HSA} : 1.51×10^{-4} M

Though the measured differences between L-Trp and D-Trp are characteristic of the ΣnK values and it is not feasible to separate parameters referring to the specific binding site, the stereoselectivity observed under our experimental conditions is smaller than those recently found for some benzodiazepines (Müller and Wollert, 1975b; Alebic-Kolbah et al., 1979a; Gratton et al., 1979; Simonyi et al., 1980a, b).

Acknowledgement

The authors are indebted to Dr. V. Jancsik for the preparation of HSA immobilized on CNBr activated Sepharose and to Dr. Zs. Tegyey for the synthesis of [^{14}C]OAc. HSA immobilized in microparticles was kindly supplied by Prof. I. Sjöholm (Uppsala). This work was supported by a project of The Hungarian Academy of Sciences.

REFERENCES

Alebic-Kolbach, T., Kajfez, F., Rendic, S., Sunjic, V., Konowal, A., Snatzke, G. (1979a) Biochem. Pharmacol. 28, 2457-2464

Alebic-Kolbach, T., Rendic, S., Fuks, Z., Sunjic, V., Kajfez, F. (1979b) Acta Pharm. Jugoslav. 29, 53-70

Bowmer, C., Lindup. W.E. (1978) Biochem. Pharmacol. 27, 937-942

Bruderlein, H., Bernstein, J. (1979) J. Biol. Chem. 254, 11570-11576

Bruderlein, H., Bernstein, J. (1980) Biochem. Pharmacol. 29, 1915-1918

Gratton, G., Rendic, S., Sunjic, V., Kajfez, F. (1979) Acta Pharm. Jugoslav. 29, 119-124

King, T.P., Spencer, M. (1970) J. Biol. Chem. 245, 6134-6148

Kragh-Hansen, U. (1981) Pharmacol. Rev. 33, 17-53

Lagercrantz, C., Larsson, T., Denfors, I. (1981) Comp. Biochem. Physiol. 69C, 375-378

McMenamy, R.H., Oncley, J.L. (1958) J. Biol. Chem. 233, 1436-1447

Monti, J.P., Sarrazin, M., Briand, C., Crevat, A. (1977) J. Chim. Phys. 74, 942-946

Müller, W.E., Wollert, U. (1975a) Naunyn-Schmiedeberg's Arch. Pharmacol. 288, 17-27

Müller, W.E., Wollert, U. (1975b) Molec. Pharmacol. 11, 52-60

Sébille, B., Thuaud, N. (1980) J. Liqu. Chrom. 3, 299-308

Simonyi, M., Fitos, I., Tegyey, Zs. (1980a) JCS. Chem. Comm. 1105-1106

Simonyi, M., Fitos, I., Tegyey, Zs., Ötvös, L. (1980b) Biochem. Biophys. Res. Comm. 97, 1-7

Sjöholm, I., Ekman, B., Kober, A., Ljungstedt-Pahlman, I., Seiving, B., Sjödin, T. (1979) Molec. Pharmacol. 16, 767-777

Tegyey. Zs., Maksay, G., Ötvös, L. (1979) J. Labelled Compounds 16, 377-385

Vallner, J.J., Perrin, J.H., Wold, S. (1976) J. Pharmacol Sci. 65, 1182-1187

Acta Biochim. Biophys. Hung. 21/3/, pp. 247-256 /1986/

EFFECT OF C-PROTEIN AND LC-LIGHT CHAINS ON ACTOMYOSIN ATPase AT VARIOUS IONIC STRENGTH AND CALCIUM LEVELS

N.A. Freydina, Z.I. Vishnevskaya, S.N. Udaltsov, Z.A. Podlubnaya
Institute of Biological Physics, Academy of Sciences USSR, Pushchino (Moscow Region), Soviet Union

(Received August 16, 1984)

Interrelation between the effects of C-protein and LC_2-light chains on actin-activated ATPase activity of skeletal muscle myosin has been investigated at various ionic strength (0.06-0.14) and free calcium levels (10^{-4} M, 10^{-8} M). The ATPase activity of AM reconstituted with column-purified myosin or partly-purified myosin and non-regulated actin exhibits a pronounced dependence on ionic strength with maximum at I = 0.1. C-protein impurities (5 per cent) usually present in M_{init} can inhibit AM ATPase at every ionic strength assayed, without changing the character of this dependence. Actin-activated ATPase of the above myosins shows calcium sensitivity at every ionic strength studied. The partial removal of LC_2 from M_{col} results in a decrease of AM ATPase and in a disappearance of its calcium sensitivity. C-protein added to M_{col} in a molar ratio of 1:1 inhibits considerably AM ATPase, reduces its sensitivity to ionic strength and abolishes its calcium sensitivity.

Abbreviations

DTNB - 5,5'-dithiobis (2-nitrobenzoic acid); DTT - dithiothreitol: EGTA - ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid; SDS - sodium dodecyl sulphate; AM - actomyosin; LC_2 - light chains-2; M_{init} - myosin partly purified with ammonium sulphate; M_{col} -column-purified myosin; M_{DTNB} - LC_2-deficient myosin; I - ionic strength; S1 - subfragment 1; S2 - subfragment 2

INTRODUCTION

It is usually accepted, that the contraction of skeletal muscles is regulated by the interaction of calcium ions with the specific protein-troponin, which is located on actin filaments (actin-linked calcium regulation) (Ebashi, Endo, 1968; Weber, Murray, 1973; Perry, 1979). However, the discovery of the myosin-linked calcium regulation in molluscan muscles (Szent-Györgyi, et al., 1973) and the dual

regulation system in muscles of most invertebrates (Lehman et al., 1972; Lehman, Szent-Györgyi, 1975) promoted the search for the second control mechanism in vertebrate skeletal muscle, involving Ca^{2+}-binding to myosin filaments.

The molecule of skeletal muscle myosin contains two light chains (LC_2), analogous to the regulatory light chains of molluscan myosin. LC_2 were shown to be capable of binding calcium ions with high affinity in the presence of physiological levels of free magnesium ion (Bremel, Weber, 1975; Alexis, Gratzer, 1978; Bagshaw, Kendrick-Jones, 1979). Data concerning the possible location of LC_2 near the S1-S2 hinge region of the myosin molecule also give basis to speculation that these chains could be involved in the control of the myosin cross-bridge activity (Weeds, Pope, 1977; Katayama, Wakabayashi, 1981). Evidence for and against some type of calcium control by myosin in vertebrate skeletal muscles is being extensively discussed in the literature (Weber, Murray, 1973; Morimoto, Harrington, 1974; Werber, Oplatka, 1974; Haselgrove, 1975; Bagshaw, Reed, 1977; Pemrick, 1977; Scholey et al., 1981). Nevertheless, this question remains unresolved, mainly because of the difficulty of detection of calcium sensitivity of actomyosin (AM) ATPase in vitro in the absence of tropomyosin-troponin complex. Calcium sensitivity of actin-activated ATPase of skeletal muscle myosin was revealed only in two experiments (Lehman, 1978; Pulliam et al., 1983) at an ionic strength of 0.12 but not at the lower ionic strength values usually used for AM ATPase assays. Unsuccessful attempts of other investigators to reveal calcium sensitivity of actin-activated ATPase of skeletal myosin are presumably explained by the loss of this ATPase as a consequence of preparative procedures or of storage (Lehman et al., 1974; Pinset-Härtström, Whalen, 1979; Chin, Rowe, 1982; Pulliam et al., 1983). There is also a possibility that the calcium sensitivity of ATPase is to some extent affected by minor proteins, the presence of which in myosin preparations is not always taken into account. In this connection C-protein, one of the minor proteins of thick filaments, is of the greatest interest. First, it was shown to influence AM ATPase activity at various ionic strength (Offer et al., 1973, Moos, Feng, 1980). Second, C-protein can bind to the S2-region as well as to the light meromyosin-one of myosin molecule (Moos et al., 1975; Starr, Offer, 1978) and therefore it may be supposed to have some influence on the cross-bridge movement along with LC_2.

The purpose of this paper is to study the interrelation between the effects of C-protein and LC_2 on actin-activated ATPase activity of skeletal myosin at various ionic stgrength and free calcium levels. We have shown, that C-protein, added to column-purified myosin in a molar ratio of 1:1, inhibits AM ATPase and abolishes its calcium sensitivity which, in the absence of C-protein but in the presence of LC_2, would not be blocked at either low or high ionic strength values. The results of this investigation have been published in abbreviated form in a paper by Freydina et al. (1982).

MATERIALS AND METHODS

Myosin and C-protein were prepared from rabbit skeletal muscle by the method of Offer et al. (1973). Myosin purified with ammonium sulphate from all impurities other than C-protein (M_{init}) and myosin separated from C-protein by column chromatography on DEAE-Sephadex a-50 (M_{col}) were used. LC_2-deficient myosin (M_{DTNB}) was prepared by treating column-purified myosin with DTNB-reagent according to Weeds and Lowey (1971) with modifications of Pemrick (1977).

For ATPase assays the myosin preparations were dialysed against 0.4 M KCl, 0.001 M DTT, 0.005 M imidazol-HCl, pH 7,0 and were clarified by centrifugation.

The C-protein peak from the column was concentrated by Sephadex G-25, dialysed against 0.3 M KCl, 0.005 M imidazol-HCl, pH 7.0 and used for recombination with M_{col} and M_{DTNB}.

Actin was extracted from aceton dried muscle powder according to Spudich and Watt (1971).

Protein concentrations were estimated spectrophotometrically (Specord UV Vis). The following extinction coefficients (cm^2/mg) at 280 nm were used: 0.55 for M_{init} and 0.52 for M_{col} (Godfrey, Harrington, 1970), 1.09 for C-protein (Offer et al., 1973). 1.09 for actin (Rees, Young, 1967).

All proteins were tested for purity by SDS polyacrylamide gel electrophoresis (Weber, Osborn, 1969; Laemmly, 1970).

ATPase assays were carried out at 25°C and constant stirring with a magnetic stirrer. Steady-state ATP-hydrolysis was followed by continuous recording of the pH-changes. The reaction was initiated by addition of preformed myosin filaments to the reaction mixture containing the necessary amounts of actin and ATP (Moos, Feng, 1980). The myosin

filaments were formed by dilution of stock myosin solution (with or without C-protein) to the required ionic conditions. The reaction mixture contained 0.3 mg/ml myosin, 0.3 mg/ml actin, 0.002 M ATP, 0.005 M $MgCl_2$, 0.005 M imidazol-HCl, pH 7.0 in the volume of 4 ml and 10^{-4} M $CaCl_2$ or 0.002 M EGTA to produce pCa 4 or pCa 8, respectively. The ionic strength of the solutions used was made up by various concentrations of KCl (from 0.04 M to 0.12 M). The contribution of the other components of media in ionic strength was of 0.02 M. The chosen range included that ionic strength values at which the calcium sensitivity of actin-activated ATPase activity of skeletal muscle myosin had previously been demonstrated (Lehman, 1978; Pulliam et al., 1983). The lower end of the range was limited by the stability of the myosin filament structure against low ionic strength, while the upper limit was set by the dissociation of the actomyosin systems at ionic strength values exceeding 0.12 (Podlubnaya, 1973).

Results

The curves of the dependence of ATPase activity of actomyosins reconstituted with pure actin and myosin with (M_{init}) and without (M_{col}) C-protein on ionic strength (I) are presented in Fig. 1. Since the AM preparations in different experiments somewhat vary in their ATPase activity levels, the data were normalized to the ATPase activity value of AM_{col} at ionic strength of 0.1 and pCa 4. As demonstrated in Fig. 1 the ATPase of each AM system exhibits a pronounced dependence on ionic strength with maxima at I=0.1. These results are in agreement with those obtained by Gachechiladze et al. (1970). It should be noted that at ionic strength values above about 0.12, a low ATPase rate is observed with either of the AM preparations; this is most probably a consequence of a dissociating action of increasing ionic strength on the AM system. As it can be seen from Fig. 1, the ATPase activity of AM_{init} is lower than that of AM_{col} at any ionic strength value assayed, i.e. even a small amount of C-protein normally present in myosin preparations exerts the inhibitory action on AM ATPase. However, neither conventional impurity (5 per cent) C-protein in M_{init}, nor the change of calcium level in the system can influence the characteristic dependence of ATPase activity on ionic strength.

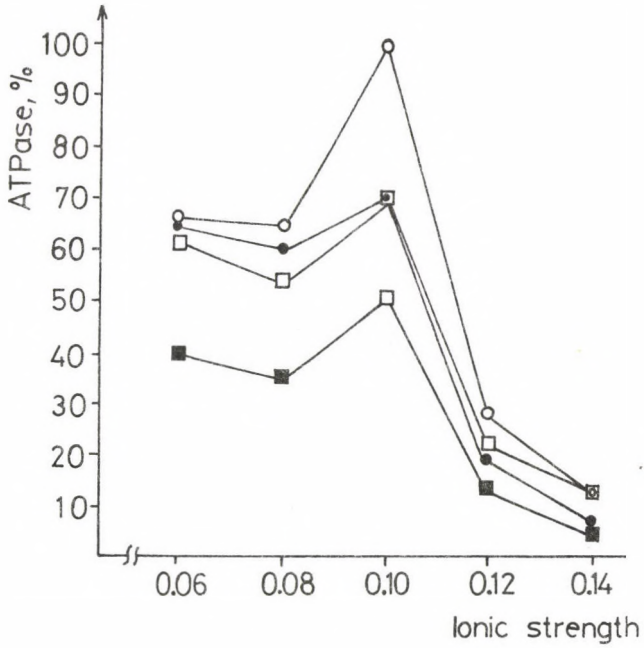


Fig. 1 Effect of ionic strength on actomyosin ATPase. □—□ partly-purified myosin, pCa 4; ■—■ partly-purified myosin, pCa 8; O—O column-purified myosin, pCa 4; ●—● column-purified myosin, pCa 8. The data are normalized to the ATPase activity value of AM_{col} at $\underline{I}$ = 0.1 and pCa 4.

The addition of C-protein to M_{col} in a molar ratio of 1:1 (Fig. 2) results in an increased inhibitory effect on AM ATPase at $I < 0.12$ and in a decreased sensitivity to ionic strength: the maximum disappears and the level of ATPase activity remains practically constant throughout the range of ionic strength values up to $\underline{I}$=0.12. At ionic strength values above 0.12 the ATPase activity of actomyosin in the presence of C-protein is somewhat higher than that in its absence (Fig. 2), which correspond to the date of Moos and Feng (1980).

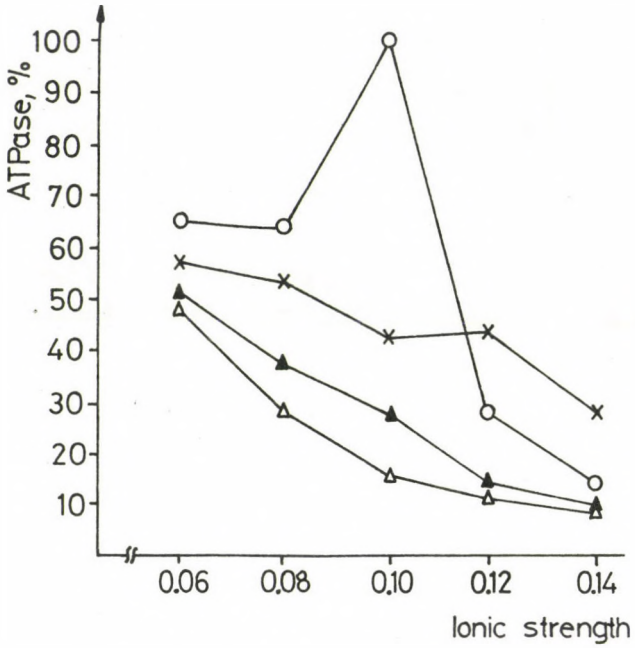


Fig.2. Effect of ionic strength on actomyosin ATPase. ○——○ column-purified myosin; ×——× column-purified myosin with added C-protein in a molar ratio of 1:1; △——△ LC_2-deficient myosin; ▲——▲ LC_2-deficient myosin with added C-protein in a molar ratio of 1:1. The data are normalized to the ATPase activity value of AM_{col} at $\underline{I}$=0.1 and pCa 4.

In a number of experiments we also observed the influence of calcium level on ATPase of reconstituted actomyosins (AM_{init} and AM_{col}), despite the fact that the actin used did not contain regulatory proteins. As seen from Fig. 1, the ATPase activity levels of the above AM systems at pCa 4 are higher than those at pCa 8 throughout the range of ionic strength values studied. The validity of difference in the AM ATPase activity levels at pCa 4 and pCa 8 is 97-99.9 per cent according to Student's criteria. The validity of difference of curves by the sign test is 95 per cent.

C-protein, added to M_{col} in a molar ratio of 1:1, inhibits calcium sensitivity of AM_{col}: practically no difference in ATPase activity depending on pCa is revealed in this case.

The partial removal of LC_2 from column-purified myosin by its treatment with DTNB -reagent causes considerable reduction of AM ATPase activity level (Fig. 2). This corresponds to the data reported by others (Werber, Oplatka, 1974; Pemrick, 1977; Hozumi, Hotta, 1978). It should be noted that the K-EDTA ATPase activity of M_{DTNB}, as compared with that of M_{col}, is slightly reduced and in some cases remains unaltered. The dependence of ATPase activity of AM_{DTNB} on ionic strength is illustrated in Fig. 2. The ATPase activity in such system decreases monotonically with the increase of ionic strength. This system, as it may be expected, is insensitive to the change in calcium-level. C-protein added to M_{DTNB} in a molar ratio of 1:1 slightly raises the level of AM ATPase activity, not changing the character of its dependence on ionic strength (Fig. 2).

Discussion

The analysis of the data on the influence of C-protein on AM ATPase shows that, even usual C-protein contamination in myosin (5 per cent) inhibits remarkably the ATPase rate in the range of ionic strength from 0.06 to 0.14 (Kakol, 1974). Addition of C-protein to M_{col} in a molar ratio of 1:1 results in an increase of this inhibitory effect. In this case the curve shape of the dependence of ATPase on ionic strength also changes: the maximum disappears and the ATPase rate is almost independent of ionic strength up to $\underline{I}$=0.14. At ionic strength values up to 0.12 the ATPase of AM_{col} in the presence of C-protein is lower than that of AM_{col}. At ionic strength values above 0.12 the level of C-protein-modified ATPase is somewhat higher than the ATPase of AM_{col} or of AM_{init} (compare Figs. 1 and 2). This fact is explained by Moos and Fend (1980) by presuming a specific activating effect of C-protein under these conditions. However, it would also be reasonable to explain this fact by a stabilizing action of C-protein on AM systems. Indeed, it is known that C-protein interacts not only with myosin filaments, but with actin filaments too, although its affinity for myosin is higher than for actin (Moos et al., 1978). It may be supposed that in our systems C-protein, when located on the surface of myosin filaments, simultaneously interacts with actin and thus enhances AM resistance to the dissociating influence of increasing ionic strength values. When the C-protein content in a system is low, its stabilizing effect is minute, almost

unobservable. However, the increase of C-protein content results in a noticable upward shift in the dissociating values of the ionic strength. If this is the case, then this "activating" effect of C-protein will exhibit itself only in an in vitro AM system, in which myosin and actin filaments can move rather freely in suspension. However, it is unlikely to be of any importance in a living muscle in which the filaments are fixed.

The partial removal of LC_2 considerably lowers the actin-activated ATPase level of M_{col} and changes the character of ATPase dependence on ionic strength. This result confirms the available data concerning the strong influence of LC_2 on actin-myosin interaction both in the presence and in the absence of ATP (Werber, Oplatka, 1974; Margossian et al., 1975; Pemrick, 1977; Hozumi, Hotta, 1978; Wikman-Coffelt et al., 1979). It is essential to note that the removal of one LC_2 from a myosin molecule results in a disappearance of maximum in ATPase activity at an ionic strength of 0.1 (Fig. 2). Addition of C-protein to M_{DTNB} in a molar ratio of 1:1 does not lead to reappearance of this maximum. It can not be excluded that the maximum is determined by the presence of two native LC_2 chains in the myosin molecule.

The question about the role of LC_2 of skeletal muscle myosin in a regulatory mechanism is widely discussed in the literature. The increase of ATPase activity of myosin in complex with nonregulated actin in the presence of calcium, as revealed by us, is another confirmation of calcium sensitivity of vertebrate skeletal muscle myosin. Contrary to the results of Pulliam et al. (1983) and of Lehman (1978), in our experiments the calcium sensitivity was demonstrated at each ionic strength studied. However, the date obtained are difficult to reproduce. Calcium sensitivity in our experiments ranged between 3 and 42 per cent. Moreover, sometimes the addition of calcium had an inhibitory effect on AM ATPase activity (see also: Weber, Murray, 1973; Pulliam et al., 1983). The explanation for this can be related to the delicacy of the myosin isolation method. Similar difficulties took place, for example, when the calcium sensitivity of insect flight muscle myosin was investigated (Lehman et al., 1974). The insect muscle myosin, Ca^{2+}-regulated in vivo, easily loses this property during its isolation from the muscle. One can think, that rabbit skeletal myosin can also lose a regulatory property which it possessed in vivo. Recently it has been shown that the loss of calcium sensitivity of skeletal muscle myosin is

due to its treatment by high ionic strength during isolation. Avoiding these treatments, Chin and Rowe (1982) could demonstrate calcium sensitivity of myosin both at physiological and at low ionic strength values. It is interesting to note that the calcium sensitivity of skeletal muscle myosin was first revealed in myosin preparations not exposed to high ionic strength before ATPase assays and, in particular, in rabbit skeletal muscle myofibrils (Lehman, 1978). At present, it is impossible to say definitely what is the true reason (or reasons) of unsuccessful calcium sensitivity assays. In the light of our results, as well as of those of other authors, one can conclude that there are many factors affecting calcium sensitivity of skeletal myosin, such as pH, ionic strength, heavy chains of myosin, phosphorylation of light chains, storage of myosin, presence of C-protein. Indeed, any of these not only modifies AM ATPase but also affects its calcium sensitivity. In our paper C-protein is shown to be able to inhibit both AM ATPase activity and its calcium sensitivity. However, it is not yet clear, whether the effects of C-protein observed in vitro are of any importance in living muscle, and if so, how they can be abolished.

Further elucidation of the factors essential for the expression of calcium sensitivity of skeletal muscle myosin is needed for understanding the mechanism of myosin-linked regulation (if any) of contractile activity of skeletal muscle.

REFERENCES

Alexis, M.N., Gratzer, W.B. (1978) Biochemistry 17 2319-2325

Bagshaw, C.R., Reed, G.H. (1977) FEBS Lett. 81 386-390

Bagshaw, C.R. Kendrick-Jones, J. (1979) J. Mol. Biol. 130 317-336

Bremel, R.D., Weber, A. (1975) Biochim. Biophys. Acta 376 366-374

Chin, T.F., Rowe, A.J. (1982) J. Muscle Res. Cell Motil. 3 118

Ebashi, S., Endo, M. (1968) Progr. Biophys. Mol. Biol. 18 123-183

Freydina, N.A., Vishnevskaya, Z.I., Udaltsov, S.N., Podlubnaya, Z.A. (1982) Proc. First All Union Biophys. Congr. Moscow, v.II, p. 32

Gachechiladze, N.A., Zaalishvili, M.M., Kurdovanidze, Ts.A. (1970) Bull. Acad. Sci. Georgian SSR 60 701-704

Godfrey, J.E., Harrington, W.F. (1970) Biochemistry 2 886-893; see also 894-908

Haselgrove, J.C. (1975) J. Mol. Biol. 92 113-143

Hozumi, T., Hotta, K. (1978) J. Biochem. 83 671-676
Kakol, I. (1974) Proc. Ninth FEBS Meet. 31 187-192
Katayama, E., Wakabayashi, T. (1981) J. Biochem. 90 703-714
Laemmly, U.K. (1970) Nature (London) 227 680-685
Lehman, W. (1978) Nature (London) 274 80-81
Lehman, W., Kendrick-Jones, J., Szent-Györgyi, A.G. (1972) Cold Spring Harbor Symp. Quant. Biol. 37 319-330
Lehman, W., Bullard, B., Hammond, K. (1974) J. Gen. Physiol. 63 553-563
Lehman, W., Szent-Györgyi, A.G. (1975) J. Gen. Physiol. 66 1-30
Margossian, S., Lowey, S., Barshop, B. (1975) Nature (London) 258 163-166
Moos, C., Feng, I.M. (1980) Biochim. Biophys. Acta 632 141-149
Moos, C., Offer, G., Starr, R., Bennett, P. (1975) J. Mol. Biol. 97 1-9
Moos, C., Mason, C.M., Besterman, J.M., Feng, I.M., Dubin, J.H. (1978) J. Mol. Biol. 124 571-586
Morimoto, K., Harrington, W. (1974) J. Mol. Biol. 88 693-709
Offer, G., Moos, C., Starr, R. (1973) J. Mol. Biol. 74 653-676
Pemrick, S.M. (1977) Biochemistry 16 4047-4054
Perry, S.V. (1979) Soc. Trans. Lond. 7 593-617
Pinset-Härström, I., Whalen, R.G. (1979) J. Mol. Biol. 134 189-197
Podlubnaya, Z.A. (1973) Biofizika 18 593-599
Pulliam, D.L., Sawina, V., Levine, R.J.C. (1983) Biochemistry 22 2324-2331
Rees, M.K., Young, M. (1967) J. Biol. Chem. 242 4449-4458
Scholey, J.M., Taylor, K.A., Kendrick-Jones, J. (1981) Biochimie 63 255-271
Spudich, J.A., Watt, S. (1971) J. Biol. Chem. 246 4866-4871
Starr, R., Offer, G. (1978) Biochem. J. 171 813-816
Szent-Györgyi, A.G., Szentkirályi, E.M., Kendrick-Jones, J. (1973) J. Mol. Biol. 74 179-203
Weber, A., Murray, J.M. (1973) Physiol. Rev. 53 612-673
Weber, C., Osborn, M. (1969) J. Biol. Chem. 244 4406-4412
Weeds, A.G., Lowey, S. (1971) J. Mol. Biol. 61 701-725
Weeds, A.G., Pope, B. (1977) J. Mol. Biol. 111 129-157
Werber, M.M., Oplatka, A. (1974) Biochem. Biophys. Res. Commun. 57 823-830
Wikman-Coffelt, J., Srivastava, S., Mason, D. (1979) Biochimie 61 1309-1314

Acta Biochim. Biophys. Hung. 21/3/ pp. 257-262 /1986/

THE ROLE OF THE UNIT OF MASS IN THE ALLOMETRIC EQUATION RELATING BODY SIZE AND METABOLIC RATE

Szilárd Donhoffer

Institute of Pathophysiology, University School of Medicine, Pécs, Hungary

(Received May 13, 1985)

The recent flare-up of the dispute about the correct exponent in the allometric equation centers or the question whether, for mammals, 2/3 of 3/4 should be applied as the exponent in the allometric term $\underline{a}.W^{\underline{b}}$ ($\underline{a}$ = mass coefficient, $\underline{W}$ = body mass, $\underline{b}$ = mass exponent) to the exclusion of the other, or whether 2/3 should be used for intra-, and 3/4 for inter-specific comparisons (Brody, 1945; Heusner, 1983; Feldman, McMahon, 1983; Wieser, 1984). The fact that the relationship between $\underline{W}^{2/3}$ and $\underline{W}^{3/4}$ (as that of any other pair of powers of $\underline{W}$) is uniquely determined by the chosen unit of $\underline{W}$ and the distance of the actual $\underline{W}$ from this unit, has been completely disregarded. If $\underline{W}$ is smaller than the unit of mass $\underline{W}^{2/3} > \underline{W}^{3/4}$, if $\underline{W}$ exceeds the unit of mass $\underline{W}^{2/3} < \underline{W}^{3/4}$ and, at unit mass, $\underline{W}^{2/3} = \underline{W}^{3/4} = \underline{W}^{\underline{b}} = 1$. Accordingly, the dispute about the "correct" exponent of $\underline{W}$ should be shelved.

The allometric equation $\underline{MR} = \frac{VO_2}{a.W^{\underline{b}}}$, relating body mass to metabolism (MR = metabolic rate, VO_2 = oxygen consumption of the animal, $\underline{W}$ = body mass, $\underline{a}$ = mass coefficient, $\underline{b}$ = mass exponent) is only a special case of many relationships in biology described by the allometric term $\underline{a}.X^{\underline{b}}$ (Brody, 1945; Zuckerman, 1950; Kleiber, 1961). The consensus that the relationship between metabolism and body mass should be described by an allometric equation, does not extend to the mass coefficient and the mass exponent, both are debated. At present, the controversy centers on mass exponent $\underline{b}$ as illustrated by two recent studies, one calling the exponent 3/4 a "statistical artifact" (Heusner, 1982), the other contending that "The exponent 3/4 for energy metabolism is not a statistical artifact" (Feldman, McMahon, 1983).

Akadémiai Kiadó, Budapest

Proposals for exponent $\underline{b}$ range from about 2/5 to 4/5, and even further (Brody, 1945; Bartels, 1982; Kayser, Heusner, 1964; Lee, 1939). Coefficient $\underline{a}$ served primarily to eliminate species differences, until Huxley (1950) defined coefficient $\underline{a}$ as the oxygen consumption (or any other variable) of an animal of unit mass. In most cases this cannot be measured experimentally because the vast majority of species do not include individuals of unit body mass, e.g. a 1 kg animal. The danger involved in extrapolation in biology has been pointed out recently by Rosen (1983). Nevertheless, the allometric equation relating metabolism and body mass has been elevated to a unique biological "law" covering mammals with body masses ranging from the 2.4 g shrew to the $3.8 \cdot 10^6$ g, i.e. 3.8 tonne elephant, and even to whales (Brody, 1945; Kleiber, 1961; Bartels, 1982).

Despite the almost general acceptance of exponent 2/3 for intra-specific and 3/4 for inter-specific studies, the true situation remains as recently summarized by Wieser (1984): "for nearly 150 years the relationship between metabolic rate and body size has exercised the minds of physiologists, but satisfactory explanation of what underlies this relationship, and even what its true form might be, seems as elusive as ever". A similar conclusion was reached by Prothero (1984) regarding the dispute about the 2/3 and 3/4 power rules: "it remains true to this day that there has been little disposition to critically examine either the data base on which the original assumptions were based or the analytical assumptions adopted".

However, even a casual glance at the conventional log-log graph relating VO_2 to $\underline{W}^{2/3}$ and $\underline{W}^{3/4}$ could have sufficed to convince biologists that the problem is not that simple as generally assumed, since the relationship of the two powers of W is reversed at $\underline{W} = 1$. Nevertheless, the postulate of Stahl (1962) that "the given equation should be invariant to arbitrary choice of scales of measurement" was without testing tacitly assumed to be complied with by the allometric equation.

Fig. 1 demonstrates the log of $\underline{W}^{\underline{b}}$ over a range of 10^6 for $\underline{W}$ and from 0 to 1 for $\underline{b}$, $\underline{W}$ being expressed in g, kg, and tonne. $\underline{W}^{\underline{b}}_{(g)}$ increases from $\underline{W} = 1$ g with increasing $\underline{W}$ and increasing $\underline{b}$, whereas

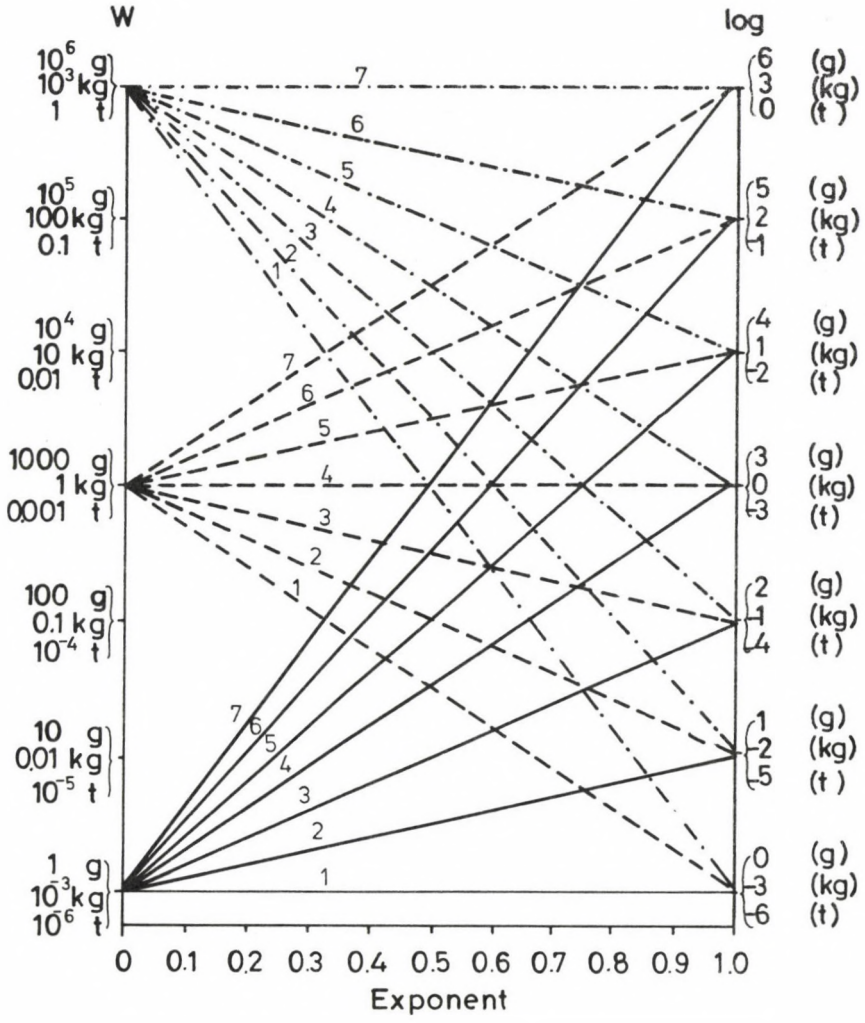


Fig. 1

$W_{(g)}$ ———	$W_{(kg)}$ -----	$W_{(t)}$ -·-·-·-
1 = 1 g	1 = 0.001 kg	1 = 10^{-6} t
2 = 10 g	2 = 0.01 kg	2 = 10^{-5} t
3 = 100 g	3 = 0.1 kg	3 = 10^{-4} t
4 = 1000 g	4 = 1 kg	4 = 10^{-3} t
5 = 10^4 g	5 = 10 kg	5 = 0.01 t
6 = 10^5 g	6 = 100 kg	6 = 0.1 t
7 = 10^6 g	7 = 1000 kg	7 = 1 t

using the tonne as unit over the same range of $\underline{W}$, i.e. from 10^{-6} to 1 t, $\underline{W}^{b}_{(t)}$ declines progressively with decreasing W and increasing $\underline{b}$. Employing the almost universally used kg as unit, $\underline{W}^{b}$ increases with $\underline{W}$ and with $\underline{b}$ if W >1 kg, and decreases with decreasing $\underline{W}$ and increasing $\underline{b}$, if $\underline{W}$< <1 kg. The explanation is, of course, quite simple: powers of bases smaller than the unit, decrease with increasing exponents and ultimately approach zero, and increase with decreasing exponents asymptotically to 1. Powers of bases greater than 1 increase, however, with increasing exponents and ultimately approach infinity, and decrease with decreasing exponents asymptotically to 1. The role of units of the measuring scale and the distance of the actual value from this unit is, of course, not confined to the case of $\underline{W}$, but applies to all equations with allometric terms of the form $\underline{X}^{b}$, e.g. length etc.

Table 1

The difference between $W^{2/3}$ and $W^{3/4}$ using different units of W

W in g	$\Delta(W^{2/3}\rightarrow W^{3/4})$ %	W in kg	$\Delta(W^{2/3}\rightarrow W^{3/4})$ %	W in t	$\Delta(W^{2/3}\rightarrow W^{3/4})$ %
1.0	0	0.001	-43.8	10^{6}	-68.4
2.5	+7.9	0.0025	-39.3	$2.5\cdot10^{-6}$	-65.9
25	+30.8	0.025	-26.5	$2.5\cdot10^{-5}$	-58.6
125	+49.5	0.125	-15.9	$1.25\cdot10^{-4}$	-52.7
500	+67.8	0.5	- 5.6	$5\cdot10^{-4}$	-46.9
750	+73.6	0.75	- 2.4	$7.5\cdot10^{-4}$	-45.1
950	+77.1	0.95	- 0.4	$9.5\cdot10^{-4}$	-44.0
1000	+77.6	1	0	0.001	-43.8
1050	+78.6	1.05	+ 0.4	0.00105	-43.5
2500	+91.9	2.5	+ 7.9	0.0025	-39.3
10000	+115.4	10	+21.1	0.01	-31.9
50000	+146.4	50	+38.5	0.05	-22.1
250000	+181.7	250	+58.0	0.25	-10.9
10^{6}	+216.2	1000	+77.8	1	$^{+}0$

Table 1 demonstrates over the same range as Fig. 1 that the choice of the unit of W, and the distance of the actual $\underline{W}$ from that unit profoundly affect the differences between $\underline{W}^{b}1$ and $\underline{W}^{b}2$ even if the difference between the two exponents is as small as between 2/3 and 3/4.

Table 2 contains results of actual observations on guinea pigs. Since in the allometric equation relating the metabolic rate of body mass, $MR = \frac{VO_2}{aW^b}$, $\underline{W}^{b}$ is in the denominator, the sign of the differences between the metabolic rates obtained with $\underline{W}^{2/3}$ and $\underline{W}^{3/4}$ are the reverse of those obtained between $\underline{W}^{2/3}$ and $\underline{W}^{3/4}$ recorded in Table 1. The same applies, of course to any other pair of powers of $\underline{W}$. Therefore, if $\underline{W}^{2/3}$ is greater than $\underline{W}^{3/4}$, the MR will be greater with $\underline{W}^{3/4}$ than with $\underline{W}^{2/3}$, or generalized, if $\underline{W}^{b}1 > \underline{W}^{b}2$, the MR calculated with $\underline{b}_2$ will exceed that gotten with $\underline{b}_1$. This is the case with the kg as the unit and $\underline{W} < 1$.

Table 2

Oxygen consumption of three groups of guinea pigs at thermoneutrality: the role of the units of mass on the relationship of results obtained with different exponents.
Mass coefficients: 10 for W in kg and 0.1 for W in g.

Group	Age (n)	Body mass M ± S.E.M.	VO_2 ml/min M ± S.E.M.	$\Delta\left(\frac{VO_2}{aW^{2/3}} \rightarrow \frac{VO_2}{aW^{2/3}}\right)$ %	$\Delta\left(\frac{VO_2}{aW^{1/2}} \rightarrow \frac{VO_2}{aW^{3/4}}\right)$ %
I	2 days (73)	92 ± 2 g 0.092 kg	2.28 ± 0.06	g -31.5 kg +22.0	-67.8 +81.6
II	30-60 d (47)	299 ± 10 g 0.299 kg	5.70 ± 0.16	g -37.8 kg +10.6	-75.9 +35.2
III	48 months (18)	820 ± 37 g 0.82 kg	10.21 ± 0.28	g -42.8 kg +1.7	-81.3 +5.1

However, if g serves as the unit, $\underline{W}^{2/3}$ is consistently smaller than $\underline{W}^{3/4}$ and, therefore, the MR calculated with $\underline{W}^{2/3}$ will be greater than that calculated with $\underline{W}^{3/4}$.

Considering that the relationship between two powers of W depends uniquely on the arbitrary choice of the unit of the measuring scale and the distance of the actual $\underline{W}$ from this unit, the controversy about the "correct" exponent and the "true" relationship between the metabolic rate and body mass makes little sense and, therefore, should be abandoned. This conclusion, inevitable as it is, does not mean that some allometric equations cannot be useful in practical work. In the course of biological interpretations, however, at least the cautious phrasing of Waddington (1950) should not be forgotten: "... the allometry equation has the status, not of a physiological principle, but of a rough and ready shorthand method of description".

REFERENCES

Bartels, H. (1982) Exptl. Biol. Med. 7 1-11

Brody, S (1945) Bioenergetics and Growth, Reinhold, New York

Feldman, H.A. McMahon, T.A. (1983) Resp. Physiol. 52 149-163

Heusner, A. (1982) Resp. Physiol. 48 1-12

Huxley, J.S. (1950) Proc. R. Soc. Lond. B. 137 465-469

Kayser, C., Heusner, A. (1964) J. Physiol. (Paris) 56 489-524

Kleiber, M. (1961) The Fire of Life, J. Wiley, New York, London

Lee, C.R. (1939) J. Nutr. 18 489-500

Prothero, J. (1984) J. Theor. Biol. 106 1-8

Rosen, R. (1983) Am. J. Physiol. 244 R591-R599

Stahl, W.R. (1962) Science 137 205-212

Waddington, C.H. (1950) Proc. R. Soc. Lond. B. 137 509-515

Wieser, W. (1984) Resp. Physiol. 55 1-10

Zuckerman, S. ed. (1950) A discussion on the measurement of growth and form. Proc. R. Soc. Lond. B. 137 433-523

Acta Biochim. Biophys. Hung. 21/3/ pp.263-281 /1986/

INVESTIGATION OF HYDRATION OF MACROMOLECULES III. STUDY OF POLYETHYLENE GLYCOL HOMOLOGUES BY MICROWAVE MEASUREMENTS

György Masszi, László Koszorus, Tibor Lakatos

Biophysical Institute, Medical University, Pécs, Hungary

(Received June 15, 1985)

The microwave conductivity and permittivity of various homologues of polyethylene glycol (PEG), as well as those of dioxane, all dissolved in either water or electrolyte solutions containing 0.01 M $MnSO_4$, were measured over the frequency range of 1.5 to 4.2 GHz, at a temperature of 30°C. The conductivity was also determined at the frequency of 20 kHz, at the same temperature. We found that

1. The wavelength of the dielectric relaxation of water increased with the increase of the concentration of non-electrolytes.
2. The orientation polarization of PEG molecules with a degree of polymerization of $\bar{n} \leq 12.5$ can be detected over the microwave range studied.
3. In solution of PEG macromolecules at a degree of polymerization $\bar{n} > 12.5$ the microviscosity and the wavelength of dielectric relaxation of water significantly increase because of the cooperative action of regularly ordered hydrophilic groups.
4. The ionic conductivity is inversely proportional to the microviscosity of the water.
5. There is a constant conductiv ity over the microwave range studied, presumably due to the orientational polarization of water molecules interacting with the macromolecules (relaxation frequency $f \approx 0.25$ to 0.5 GHz).
6. The effect of PEG of a high polimerization degree on the structure of water is similar to the effect of a decreasing temperature.

INTRODUCTION

The water binding properties of polyvynil alcohol and 1-4 dioxane have been investigated previously (Masszi et al., 1976; Koszorus, Masszi, 1982). We have found that the average wavelength of dielectric relaxation of water is greater in solutions of non-electrolytes than it is in pure

Akadémiai Kiadó, Budapest

water and the microviscosity of the water is proportional to the relaxation time.

As it has been recently reported (e.g. by Kaatze, Wen, 1978; Clegg et al., 1982; Gough, 1982; Foster et al., 1984; Liaschenko et al., 1984), various mathematical models can be fitted to a population of data measured over a wide frequency range of 0.01 to 50 GHz.

In this work the dielectric dispersion of water was studied under various experimental conditions in PEG (polyethylene glycol, $OH-(CH_2-CH_2-O)_n-H$) solutions of polymerization degrees of $\bar{n}$ = 1 to 454.5, over a frequency range of 1.5 to 4.2 GHz.

MATERIALS AND METHODS

The data of PEG homologues used in our experiments are shown in Table 1. The weight volume (w/v) ratios of the solutes/solvents were: 0.05, 0,10, 0.15, 0.20, 0.25, and 0.30. Water purified by ion exchanger and 0.01 M $MnSO_4$ water-solution were used as solvents. The purity of solutions was checked by measuring their conductivity at a frequency of 20 kHz. The conductivity of the water purified by ion exchanger was smaller than 10^{-3} ohm^{-1} m^{-1} (0.01 $mScm^{-1}$) and the PEG solutions had a conductance smaller than 3.10^{-3} $ohm^{-1}m^{-1}$ (0.03 $mScm^{-1}$).

The density of each solution was determined by picnometer and it was found to be proportional to the concentration of the non-electrolyte solute. The apparent mass densities (Fig. 1) and specific volumes (Table 1) were calculated. The 24 measurements were made at a temperature of 30°C. The apparent specific volume was constant within an error of 2 per cent over a concentration range of 0.05-0.30 w/v. The dependence of the dielectric parameters on the volume fraction ($V_{solutes}/V_{solvent}$) ϕ was studied.

The dielectric constant and conductance were determined over a frequency range of 1.5 to 4.2 GHz, at 30°C with a dielectric microviscosimeter described elsewhere (Masszi, Örkényi, 1967; Koszorus, Masszi, 1982).

The wavelengths of the dielectric relaxation of the solutions (λ_s) ("Sprungwellenlänge") were determined by three different mathematical procedures.

Table 1

The data of substances used in our experiments; M is the molecular mass, $\bar{n}$ is the average degree of polymerization. The conductances of solutions of various PEGs were smaller then 0.03 $mScm^{-1}$.

Substances		Molecular mass in atomic mass units M	$\bar{n}$	Manufactural supplier	Purity	Specific volume in solution cm^3/g ± 2 %
Ethylene glycol		62.07	1	Reanal	pro.anal	0.873
Diethylene glycol		106.12	2	Fine Chem.prod.	pract.	0.877
Triethylene glycol		150.17	3	VEB	purum	0.880
Polyethylene glycol	200	190 - 210	4.5	Fluka A.G.	pract.	0.855
"	550	550	12.5	Reanal	"	0.841
"	600	570 - 630	13.6	Fluka A.G.	"	0.840
"	1000	950 - 1050	22.7	"	"	0.824
"	2000	1900 - 2200	45.5	"	"	0.837
"	4000	3500 - 4500	90.9	"	"	0.837
"	6000	6000 - 7500	136	"	"	0.831
"	20000	17000 - 20000	452	"	"	0.837
1-4 Dioxane		88.11	2	Reanal	"	0.934

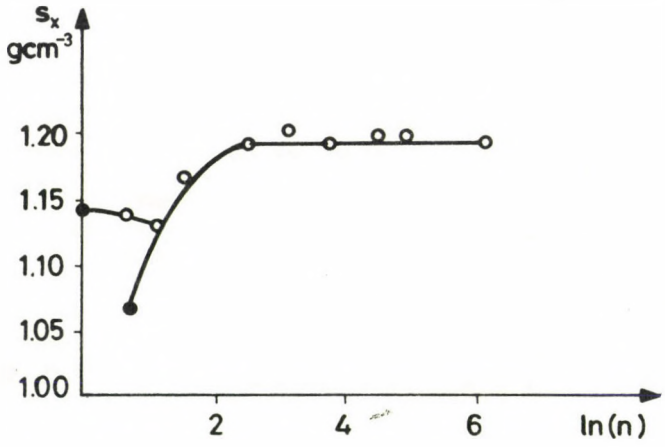


Fig. 1. The density of 1-4 dioxane (●), ethylene glycol and polyethelyne glycols (o) in diluted solution as a function of the degree of polymerization ($\bar{n}$).

1./ The equation of Debye (1929) on the dielectric constant and the conductance

$$\varepsilon' = \frac{\varepsilon_0 - \varepsilon_\infty}{1 + \left(\frac{\lambda_s}{\lambda}\right)^2} + \varepsilon_\infty \qquad (1)$$

$$\sigma = \frac{f}{1.8} \cdot \frac{\varepsilon_0 - \varepsilon_\infty}{1 + \left(\frac{\lambda_s}{\lambda}\right)^2} \cdot \frac{\lambda_s}{\lambda} + \sigma_{0m} \; |\mathrm{mScm^{-1}}| \qquad (2)$$

where ε' is the dielectric constant (permittivity)

ε_o is the static dielectric constant

ε_∞ is the dielectric constant at wavelengths much shorter than the wavelength of relaxation.

λ_s is the wavelength of relaxation.

$$\lambda_s = \frac{c}{2 \cdot \pi \cdot \tau}$$

$$\lambda_s = \frac{c}{f_c}$$

where c is the velocity of light

τ is the relaxation time

f_c is the frequency of relaxation

f is the frequency in GHz units

σ is the conductivity in $mScm^{-1}$ units

σ_{0m} is the constant component of the conductivity within the range of the microwaves

When $\lambda_s \ll \lambda$, then from equation 2 it follows that:

$$\sigma = \frac{f}{1.8} \cdot \frac{\varepsilon_0 - \varepsilon_\infty}{\lambda} \cdot \lambda_s + \sigma_{0m} \quad |mScm^{-1}| \qquad (3)$$

$$\sigma = \frac{(\varepsilon_0 - \varepsilon_\infty) \cdot \lambda_s}{54} \cdot f^2 + \sigma_{0m} \quad |mScm^{-1}| \qquad (4)$$

Plotted against f^2, σ will result in a straight line. The "relaxation wavelength for a long wavelength" (Budó, 1949) λ_s can be determined from the slope and the σ_{0m} intersection of the straight line, respectively. The relaxation wavelength of water at 30°C is 1.36 cm (Hasted, 1961), therefore the condition $\lambda_s \ll \lambda$ is satisfied over a range of λ = 7 to 20 cm. The value of ε_0 is estimated from equation 1. The concentration - when $\phi \leq 0.3$ - will not influence significantly the value of ε_∞ (e.g. Kaatze et al., 1978), therefore we used $\varepsilon_\infty = 4.2$ (Franks, 1972) in our calculations. The method is not only simple, but it results in the weighted mean of the relaxation wavelengths in the case of compartmentalized systems which have a number of different relaxation wavelengths λ_{s1}, λ_{s2}, λ_{s3} ... λ_{si} (e.g. Katona, Vasilescu, 1984), or in the case of a few layers of water (e.g. Ling, Murmphy, 1983; Ling, 1984). The component wavelengths are weighted by the change of the

dielectric constant (Masszi, 1972):

$$\lambda_s = \frac{\sum_i \Delta\varepsilon_i \cdot \lambda_{s_i}}{\Delta\varepsilon} \qquad (5)$$

where $\Delta\varepsilon_i$ is the decrease of the dielectric constant of the i^{th} component withhin the range of relaxation.

$\Delta\varepsilon$ is the total decrease of the dielectric constant over the range of relaxation.

2./ As derived from Debye's equation:

$$\frac{f}{\sigma - \sigma_{0m}} = \frac{54 \cdot \lambda_s}{900 \cdot (\varepsilon_0 - \varepsilon_\infty)} \cdot f^2 + \frac{54}{(\varepsilon_0 - \varepsilon_\infty) \cdot \lambda_s} \qquad (6)$$

We determined with computer that value of σ_{om} at which the sum of the squared deviations is minimum for the function

$$\frac{f}{\sigma - \sigma_{0m}} = k_1 \cdot f^2 + k_2 \qquad (7)$$

The constants k_1 and k_2 were determined, too. From equations /6/ and /7/:

$$\lambda_s = \frac{900 \cdot (\varepsilon_0 - \varepsilon_\infty) \cdot k_1}{54} \qquad (8)$$

and

$$\lambda_s = \frac{54}{(\varepsilon_0 - \varepsilon_\infty) \cdot k_2} \qquad (9)$$

$$\lambda_s = 30 \cdot \sqrt{\frac{k_1}{k_2}} \qquad (10)$$

The difference between the wavelengths of relaxation as calculated from equations /8/ and /9/ is about three per cent.

3./ According to the complete Cole and Cole equation (Cole, Cole, 1941):

$$\sigma = \frac{\varepsilon_0 - \varepsilon_\infty}{1.8} \cdot \frac{f^{2-\alpha} \cdot (\varepsilon_0 - \varepsilon_\infty) \cdot \cos\frac{\alpha \cdot \pi}{2}}{f^{1-\alpha} + 2 \cdot f^{1-\alpha} \cdot \sin\frac{\alpha \cdot \pi}{2} + f^{1-\alpha} \cdot \left(\frac{f}{f_c}\right)^{2 \cdot (1-\alpha)}} + \sigma_{0m} \quad |\mathrm{mScm}^{-1}| \qquad (11)$$

Table 2

Parameters determined by Cole-Cole fitting, α is the spread parameter, σ_{om} is the constant conductivity over the microwave range, σ_o is the conductivity measured at the frequency of 20 kHz.

Polyethylene glycol 6000 $\phi = 0.27$

	α	σ_{om} mScm^{-1}	σ_o mScm^{-1}
Polyethylene glycol + H_2O	0.05	3.34	0.03
Polyethylene glycol + 0.01 M $MnSO_4$	0.01	4.04	0.37
$\Delta\sigma$		0.70	0.34

Polyethylene glycol 20000 $\phi = 0.27$

	α	σ_{om} mScm^{-1}	σ_o mScm^{-1}
Polyethylene glycol + H_2O	0.18	0.03	0.07
Polyethylene glycol + 0.01 M $MnSO_4$	0.21	0.05	0.44
$\Delta\sigma$		0.02	0.37

where α is the spread parameter of relaxation times.

The parameters $(\varepsilon_0 - \varepsilon_\infty)$, λ_s, α, and σ_{om} were determined with computer by a fitting program, when the sum of the squared deviation from function /11/ was minimum. We have found that α increased when σ_{om} decreased. In all cases, when the conductance of a PEG homologue solution containing 0.01 molar $MnSO_4$ was greater than that of a similar solution without electrolyte, and the excess corresponded to the conductance at 20 kHz, α was about zero and the solution without electrolyte had a σ_{om} which was greater than the conductance measured at audio frequencies (Table 2). The values of both $(\varepsilon_0 - \varepsilon_\infty)$ and λ_s coincide with those determined by the two previous methods. Our results have revealed that the four-parametric fitting is too sensitive to determine α and σ_{om} within the initial range of dispersion. The data of solutions containing electrolyte suggested that $\alpha < 0.1$ and σ_{om} found for solutions without electrolyte may be due to a dispersion having a frequency of relaxation $f_c < 1.0$ GHz.[x]

RESULTS

1. The dependence of the static dielectric constant (ε_0) on the volume fraction ϕ of non-electrolyte solutes in the solvent water 0.01 M $MnSO_4$ and the average degree of polymerization $\bar{n}$ are shown in Fig. 2. As it is demonstrated, the dielectric constant is independent of the degree of polymerization and it is equal to the dielectric constant of dioxane when $\bar{n} > 12.5$. We concluded from this that PEG molecules, at a degree of polymerization $\bar{n} > 12.5$, have no rotation over the microwave range and they do not contribute to the orientation polarization of the solution, similarly to the nonpolar molecules of dioxane.
However, the orientation polarization of the solute does contribute to the microwave dielectric constant when the degree of polarization is

[x]It we use the most recent, precise data (Steel et al., 1984) concerning the microwave dielectric parameters of the lens cortex of rabbit, at 20°C, over a frequency range of 3.81 to 18,0 GHz, the third method mentioned above produces the next results: $\alpha = 0.03$, $\sigma_{om} = 6.46$ mScm^{-1}, $\varepsilon_0 = 49.9$, $\lambda_s = 2.68$ cm ($\lambda_{s_{H_2O}} = 1.75$ cm)

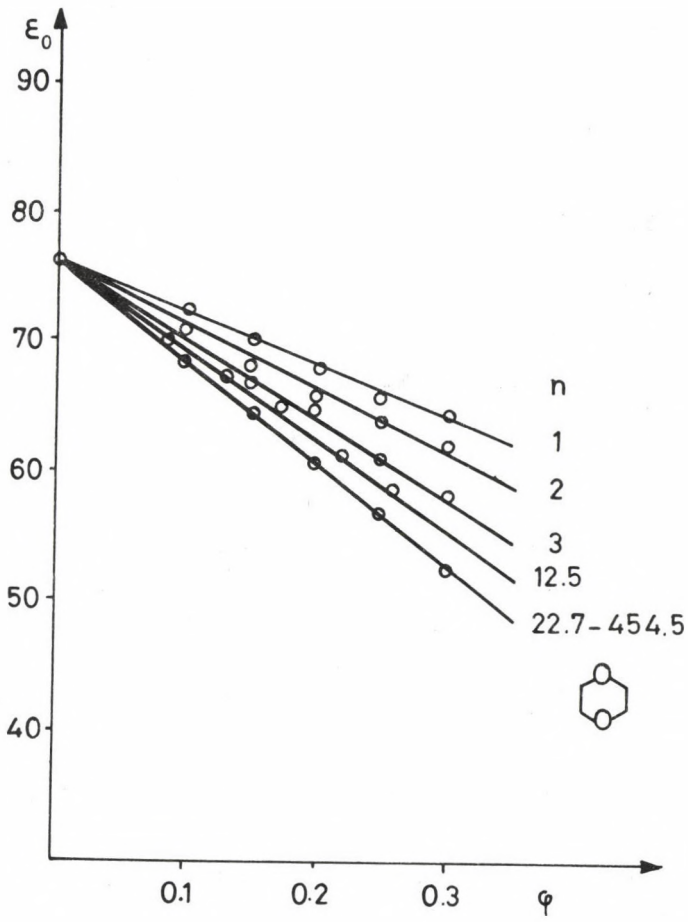


Fig. 2 The dependence of ε_0 on volume fraction in water and in 0.01 M $MnSO_4$ solutions.

$\bar{n} \leq 12.5$; the smaller the molecule, the greater the contribution. As it is shown in Fig. 3, the dielectric decrement $-\frac{\Delta\varepsilon_0}{\Delta\phi}$ increased with increasing degrees of polymerization and it reached the dielectric decrement of the 1-4 dioxane at $\bar{n} = 22.7$.

2. The wavelength of relaxation depends on both the concentration of solute and the degree of polymerization, as shown in Fig. 4.

The wavelength of relaxation is constant and it is independent of the degree of polymerization when $\bar{n}$ is greater than 22.7, similarly to the dielectric constant. However, the relaxation wavelengths of 1-4 dioxane solutions are smaller than those of PEG solutions of the same concentrations. It proves that the dipole rotation of water in PEG

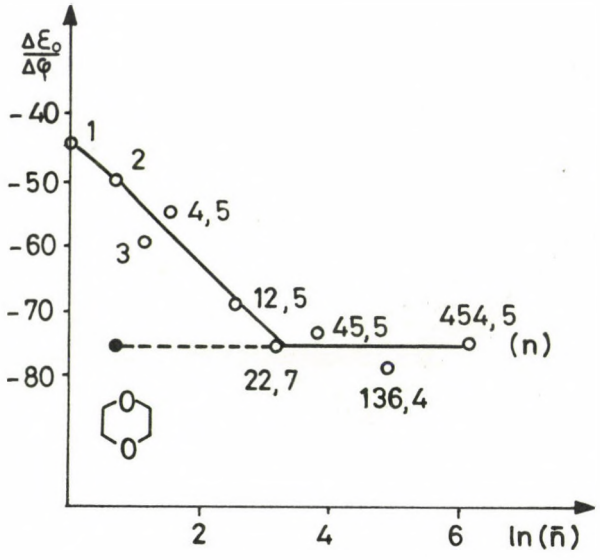


Fig. 3 The slopes of the straight lines in Fig. 2. $\frac{\Delta\varepsilon_0}{\Delta\Phi_0}$ in dioxane, ethylene glycol and polyethylene glycol solutions as a function of the degree of polymerization $\bar{n}$ in water and in 0.01 M $MnSO_4$ solutions.

solutions is more restricted than it is in dioxane solutions of the same dielectric constant.

The relaxation wavelength of PEG homologues (λ_{s_e}) of $\bar{n} < 22.7$ was determined from equation /5/:

$$\lambda_s = \frac{\Delta\varepsilon_e \cdot \lambda_{s_e} + \Delta\varepsilon_d \cdot \lambda_{s_d}}{\Delta\varepsilon_e + \Delta\varepsilon_d} \qquad (12)$$

where λ_{s_d} is the relaxation wavelength of the dioxane solution, and $\Delta\varepsilon_d = \varepsilon_d - \varepsilon_{\infty_d}$ is the same for dioxane.
It is reasonable approximation to use the parameters of the dioxane, because the PEG molecules of small molecular mass presumably influence the dielectric properties of water in the same way, as the 1-4 dioxane of similar structure but of negligible dipole moment does.
$\Delta\varepsilon_e = \varepsilon_e - \varepsilon_d$ is the contribution of the dipole rotation of PEGs to the dielectric constant, λ_{s_e} is the relaxation wavelength of PEGs, λ_s is the average relaxation wavelength of the solution. Considering that the dielectric constant decreases linearly over the

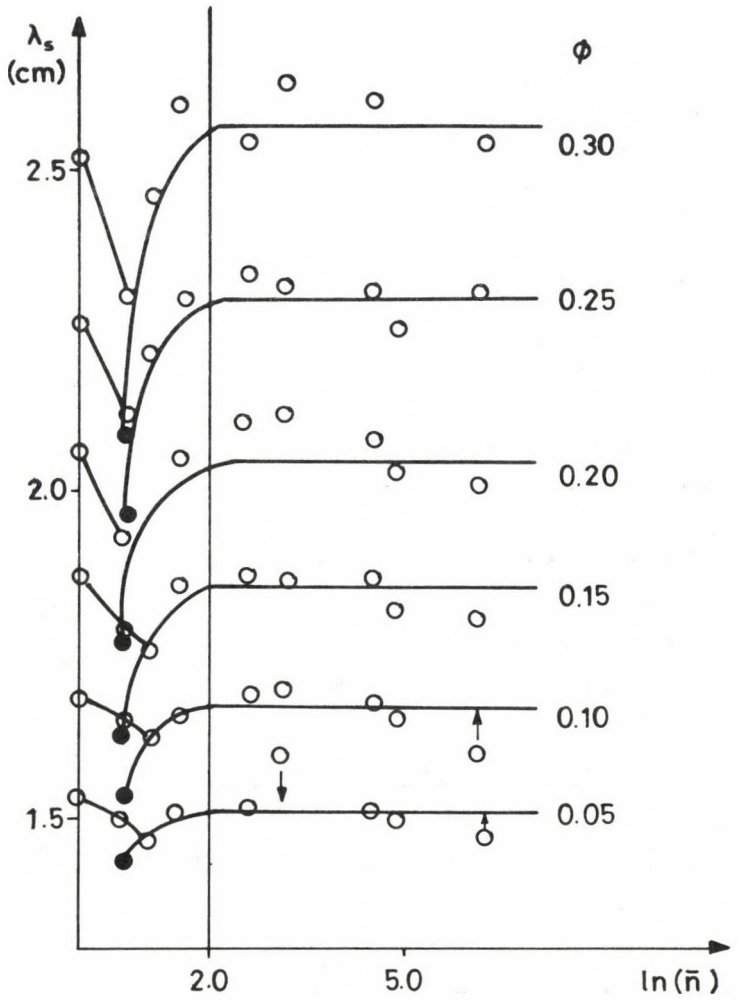


Fig. 4 The relaxation wavelengths of dioxane (•), ethyleneglycol and polyethylene glycol (o) solutions as functions of the degree of polymerization $\bar{n}$, in solutions of different volume fraction (ϕ), in water and in 0.01 M $MnSO_4$ solutions.

range of concentration in question (Fig. 2) and the relaxation wavelength increases linearly over the range of volume fraction 0 to 0.20 when the concentration increases, we may write:

$$\Delta\varepsilon_d = -c_d \cdot \phi + \varepsilon_0 - \varepsilon_\infty \qquad \lambda_s = k_s \cdot \phi + \lambda_{s_0}$$

$$\Delta\varepsilon_e = (c_d - c_e) \cdot \phi \qquad \lambda_{s_d} = k_d \cdot \phi + \lambda_{s_0} \qquad (13)$$

where λ_{so} is the relaxation wavelength of water. The slopes of the respective straight lines are:

$$c_d = -\frac{\Delta\varepsilon_{0_d}}{\Delta\phi}$$

$$c_e = -\frac{\Delta\varepsilon_{0_e}}{\Delta\phi}$$

$$k_s = \frac{\Delta\lambda_s}{\Delta\phi}$$

$$k_d = \frac{\Delta\lambda_{s_d}}{\Delta\phi}$$

Combination of equations 12 and 13 results in a form that describes the relaxation wavelength of PEGs ($\bar{n} \leq 13.6$):

$$\lambda_{s_e} = \frac{k_d \cdot c_d - k_e \cdot c_e}{c_d - c_e} \cdot \phi + \frac{k_e - k_d}{c_d - c_e} \cdot \lambda_{s_{H_2O}} \qquad (14)$$

The relaxation wavelength of PEGs in "infinite dilution" was calculated from this equation when zero was substituted for ϕ (Table 3).

Table 3

The relaxation wavelength of polyethylene glycols with a degree of polymerization $\bar{n} \leq 13.6$, at $\phi = 0$, is represented by λ_{s_e}, $-\frac{\Delta\varepsilon_0}{\Delta\phi}$ is the dielectric decrement $\frac{\Delta\lambda_s}{\Delta\phi}$ is the slope of the function $\lambda_s(\phi)$.

$\bar{n}$	$-\frac{\Delta\varepsilon_0}{\Delta\phi}$	$\frac{\Delta\lambda_s}{\Delta\phi}$	λ_{s_e} (cm)
1	40.5	3.3	3.3
2	49.7	2.9	2.8
3	59.5	3.1	4.5
4.5	54.4	3.5	5.1
13.6	50.6	3.7	5.2
Dioxane	77.8	2.3	

3. The viscosity of water is proportional to the relaxation wavelength of water as predicted by Debye's theory (1929) and found in microwave measurements (Collie et al., 1948; Rizk, Girgis, 1969; Grant, 1982). As it was reported previously (Masszi, 1972a; Foster et al., 1982; Koszorus, Masszi, 1982; Foster et al., 1984) the microviscosity of water is proportional to the average relaxation wavelength of water in solutions and the mobility of ions decreases according to Walden's rule (Westphal, 1928). The dependence of the ionic conductivity on the concentration in solutions of gelatine of a volume fraction of ϕ is described by the equation:

$$\frac{\sigma(\phi)}{\sigma(\phi=0)} = (1 - k \cdot \phi) \cdot \frac{\lambda_{s_{H_2O}}}{\lambda_s} \qquad (15)$$

Table 4

The dependence of relative conductivity on the volume ratio in polyethylene glycol solutions with a degree of polymerization $\bar{n} = 454.5$. Column 2: values calculated on the basis of Fricke's equation, Column 3: values calculated by taking the microviscosity into consideration. Column 4: relative conductivity values measured at 20 kHz. $\sigma(\phi = 0)$ is the conductivity of pure electrolyte (0.01 M $MnSO_4$) solution, $\sigma(\phi = 0) = (1.86 \pm 0.08)$ mScm^{-1}.

ϕ	$1-1.5\phi$	$(1-1.5\phi)\frac{\lambda_{s_{H_2O}}}{\lambda_s}$	$\frac{\sigma(\phi)}{\sigma(\phi=0)}$
0.042	0.94	0.86	0.89
0.085	0.87	0.74	0.76
0.128	0.81	0.63	0.65
0.172	0.74	0.52	0.54
0.216	0.68	0.44	0.44
0.260	0.61	0.36	0.32

This has been found in microwave measurements (Masszi, 1972), in which $\sigma(\phi)$ is the conductivity of a solution containing a non-electrolyte solute of a volume fraction of ϕ. $\underline{k}$ is a coefficient, the magnitude of which depends on the shape of the molecule (Robinson, Stokes, 1959), λ_s is the relaxation wavelength of water in the solution. The conductivity of solutions of PEG (molecular mass: 20,000) containing $MnSO_4$ of a concentration of 0.01 mol/dm^3 measured at a frequency of 20 kHz, and the parameters calculated from equation 15 are summarized in Table 4. It appears that the microviscosity of water has a significant effect on the mobility of ions.

4. The conductance σ_{om} of solutions of PEG of a degree of polimerization of $\bar{n} > 12.5$ is plotted against concentration in Fig. 5.

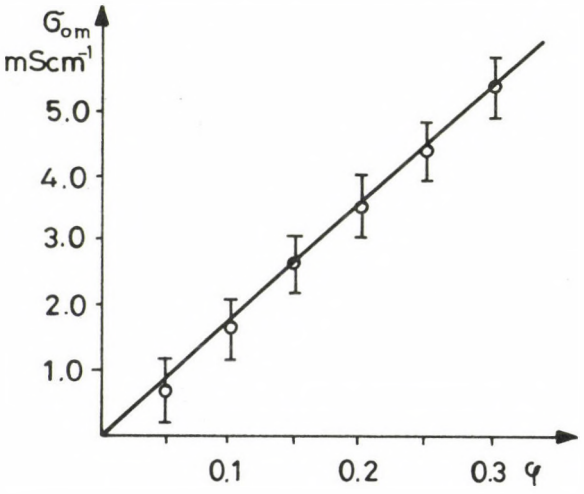


Fig. 5 The dependence of the constant conductivity (σ_{om}) in the microwave range in polyethylene glycol solutions with the degree of polymerization $\bar{n} > 12.5$. The lengths of vertical bars are equal to the double standard deviations.

Since the conductance of solutions without $MnSO_4$ at 20 kHz is smaller than 0.03 mScm^{-1}, the conductance σ_{om} is, presumably, due to the relaxation of water molecules in direct interaction with the macromolecule at frequencies $f_c < 1.0$ GHz. The equation fitted to the data and represented by Fig. 5 is:

$$\sigma_{om} = 18.8 \cdot \phi \quad |\text{mScm}^{-1}|. \qquad (16)$$

According to the Debye equation (1929):

$$\sigma_{0m} = \frac{\varepsilon_0 - \varepsilon_\infty}{1.8} \cdot f_c \quad |\mathrm{mScm}^{-1}| \qquad (17)$$

when $f \gg f_c$ and f_c is in GHz units.

The experimental determination of the dielectric decrement of water molecules interacting directly with the macromolecules encounters great difficulties. The density of water in muscles of low water content is 1.3 g/cm^3 (Pócsik, 1966). As a consequence of this, its dielectric constant differs from that of the bulk water. The dielectric constant of the mixture of lysozyme enzyme, air and water (0,3 g water in each gram of lysozyme) is ε_0 = 10 to 16 (Harvey, Hoekstra, 1972). The most probable value for the dielectric constant of the water bound directly to the haemoglobin molecule is ε_0 = 80 to 100 (Pennock, Schwan, 1969). In our experiments made with gelatin solutions (Masszi, 1970), ε_0 = 120 to 130 values have been found, and the frequency of relaxation of water bound directly to the macromolecule from equation 17 was f = 0.25 to 0.5 GHz.

DISCUSSION

1. Ernst has shown that the muscle function is related to some crystallization process (Ernst et al., 1951; Ernst, 1963). We have studied macromolecules containing hydrophilic groups at regular intervals, to clear up the physical details of this crystallization (Masszi et al., 1976). As we have found that PEG at a degree of polymerization of $\bar{n} > 12.5$ increases the relaxation wavelength of water, a cooperative interaction between the water and the macromolecule may be assumed.

Polyethylene glycol is a sort of "-NO-NO-" molecule (- negative, neutral - negative, neutral -), as termed by Ling (Ling et al., 1983; Ling, 1984), that has a meandering form in aqueous solution (Staudinger, 1947; Florin et al., 1984). The meandering shape creates water layers of alternating orientation (Fig. 6) and it strengthens the structure of water, i.e. increases the microviscosity of water.

The relaxation wavelength of water in a solution containing PEG is greater than that of pure water, as witnessed by our experiments.

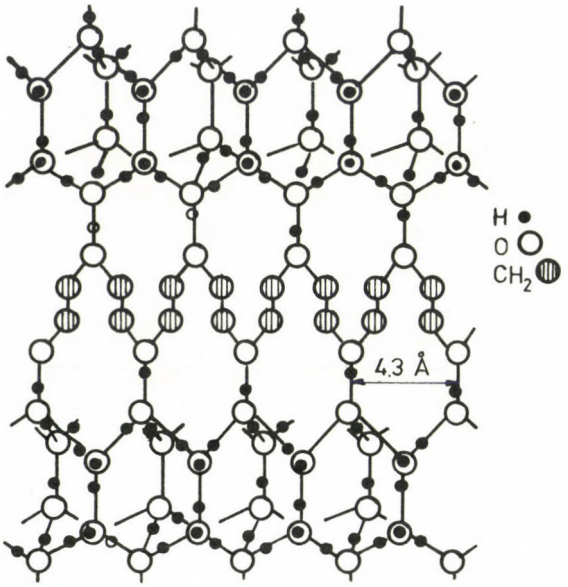


Fig. 6 The relation of polyethylene glycol to the structure of water (After Staudinger, 1947 and Bernal, Fowler, 1933).

However, further investigation are necessary to find out if this change of the relaxation wavelength is similar to the change caused by a decrease of the temperature of water, i.e. it is accompanied by a negligable spread only /Franks, 1972), or the measured wavelength of relaxation is a mean with a significant standard deviation.

2. The mechanism of relaxation processes in the bulk water is fundamentally different from the orientation processes of water molecules, bound to macromolecules through H-bonds (e.g. Luck, 1985 and Muzsnay, 1984).

In the first case, the dielectric polarization is related to the structure of water. The wavelength of relaxation is changed through the modification of that structure by the macromolecules. A recent theory (Gaiduk, Kalmykov, 1981) considers the vibrational states over the infrared range as of fundamental significance in the processes of dielectric relaxation of water. The process of relaxation may be described by the "liberations" allowed by the structure of water. The theory on the one hand, calls our attention to the significance of micro-

wave and of infrared radiations in biology (e.g. Predmerszky et al., 1982; Fröhlich, Kremer, 1983), while, on the other hand, it eliminates the contradiction in Debye's theory, i.e. that the attenuation constant of water becomes zero at frequencies high enough (e.g. Illinger, 1980).

In the second case, the orientation of a single bound water molecule may be compared to the orientation of the mobile side groups of macromolecules. The experimental discrimination of these two processes is rather difficult (Schwan, 1957; Grant et al., 1968; Essex et al., 1977; Gough, 1983), therefore it is questionable to accept the dielectric constant of single bound water molecules as a phenomenologic parameter. The bound water molecules may play a great role in the electret-like behaviour of macromolecular systems, as it has been suggested by Mikola (1925) and Mascarenhas (1975).

Acknowledgement

We are grateful to the Microwave Laboratory of the Research Institute of Telecommunication for their help, to Z. Futó, M. Kende, J. Örkényi, K.R. Foster and V.J. Gajduk for the invaluable discussions, to professors J. Tigyi and A. Niedetzky for their support, and to A. Domokos-Páll for her technical assistance.

REFERENCES

Bernal, J. D., Fowler, R.H. (1933) J. Chem. Phys. 1 515, In: Samoilov, O.J. (1961) Die Struktur von Wässrigen Electrolyt Lösungen, Leipzig, pp. 36-38

Budó, A. (1949) J. Chem. Phys. 17 686-691

Clegg, J.S., Szwarnowski, S., McClean, V.E.R., Sheppard, R.J., Grant, E.H. (1982) Biochim. Biophys. Acta 721 458-468

Cole, H., Cole, K.S. (1941) J. Chem. Phys. 9 341-351

Collie, C.H., Hasted, J.B., Ritson, D.M. (1948) Proc. Phys. Soc. London 60 145-160

Debye, P. (1929) Polare Molekeln, Verlag Striztel, Leipzig, p. 96.

Ernst, E., Balog, J., Tigyi, J., Sebes, A. (1951) Acta Physiol. Acad. Sci. Hung. 2 253-259

Ernst, E., (1963) Biophysics of the Striated Muscle. Publ. House Acad. Sci. Budapest, p. 249

Essex, C.G., Symonds, M.S., Sheppard, R.J., Grant, E.H., Lamote, R., Soetewey, F., Rosseneu, M.Y., Peeters, M. (1977) Phys. Med. Biol. 22 1160-1167

Florin, E., Kjellander, R., Eriksson, J.Ch. (1984) J. Chem. Soc. Faraday Trans. 80 2889-2910

Foster, K.R., Cheever, E., Leonard, J.B., Blum, F.D. (1984) Biophys. J. 45 975-984

Foster, K.R., Epstein, B.R., Jenin, P.C., Mackay, R.H. (1982) J. Coll Interface Sci. 88 233-246

Franks, F. ed. (1972) Water: a Comprehensive Treatise Vol. 1. Chap. 7. Hasted, J.B., Liquid Water: Dielectric Properties P. 277, Plenum Press, New York, London

Fröhlich, H., Kremer, F. (1983) Coherent excitations in biological systems. Springer Verlag, Berlin, Heidelberg, New York, Tokyo

Gaiduk, V,J., Kalmykov, Y.P. (1981) J. Chem. Soc. Faraday Trans. 77 929-946

Gough, S.R. (1983) J. Sol. Chem. 12 729-739

Grant, E.H. (1982) Bioelectromagnetics 3 17-24

Grant, E.H., Keefe, S.E., Takashima, S. (1968) J. Phys. Chem. 72 4373-4380

Harvey, S.C., Hoekstra, P. (1972) J. Phys. Chem. 76 2987-2994

Hasted, J.B. (1961) Progr. Dielectr. 3 103-149

Illinger, K.H. (1976) In: Biological Effects of Electromagnetic Waves, HEW Publ. FDA 77-8011 pp. 169-182

Kaatze, U., Göttmann, O., Pdbielski, R., Pottel, R., Treveer, U. (1978) J. Phys. Chem. 82 112-120

Kaatze, U., Wen, W.Y. (1978) J. Phys. Chem. 82 109-111

Katona, E., Vasilescu, V. (1984) In: New trends in the study of water and ions in biological systems. Proc. Roman-American Workshop, ed. Vasilescu, V., Hazlewood, C.F., Bucharest, pp. 177-190

Koszorus, L., Masszi, G. (1982) Acta Biochim. Biophys. Acad. Sci. Hung. 17 237-249

Ling, G.N. (1984) In Search of the Physical Basis of Life. Plenum Publ. Corp. New York, London, p. 168

Ling, G.N., Murphy, R.C. (1983) Physiol. Chem. Phys. Med. NMR 15 137-153

Luck, W.A.P. (1985) In: Water and Ions in Biological Systems. Plenum Press, New York- London, pp. 269-275.

Lyashchenko, A.K., Kahrkin, V.S., Goncharov, V.S., Yastermskii, P.S. (1984) Zh. Fiz. Khim. SSSR 10 2494-2499

Mascarenhas, S. (1975) An. Acad. brasil Cience, 47 223-226

Masszi, G. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. 5 321-331

Masszi, G. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. 7 341-347

Masszi, G. (1972a) Acta Biochim. Biophys. Acad. Sci. Hung. 7 349-357

Masszi, G., Inzelt, G., Gróf, P. (1976) Acta Biochim. Biophys. Acad. Sci. Hung. 11 45-50

Masszi, G., Örkényi, J. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. 2 69-78

Mikola, S. (1925) Z. f. Phys. 32 476-488

Muzsnay, Cs. (1984) Studia Univ. Babes-Bolyai XXIX. 49-56

Pennock, B.E., Schwan, H.P. (1969) J. Phys. Chem. 73 2600-2610

Pócsik, S. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. 2 149-160

Predmerszky, T., Ballay, L., Bölöni, E., Szabó, L.D., Vámos, L. (1982) Acta Physiol. Acad. Sci. Hung. 52 479-486

Rizk, H.A., Girgis, Y.M. (1969) Z. f. Phys. Chem. Neue Folge 65 261-275

Robinson, R.A., Stokes, R.M. (1959) Electrolyte solutions, Butterworths London p. 309

Schwan, H.P. (1957) Adv. Biol. Med. Phys. V. 147-209

Staudinger, H. (1947) Makromolekulare Chemie und Biologie, Wepf Co. Verlag, Basel p. 108

Steel, M., Sheppard, R.J., Grant,E.M. (1984) J. Phys. E. Sci. Instrum. 17 pp. 30-34

Westphal, W. ed. (1928) Handbuch der Physik. Band XIII. Kap. 13. E. Baars: Elektrizitätsleitung in Flüssigkeiten, p. 440. J. Springer Verlag, Berlin

Acta Biochim. Biophys. Hung. 21/3/ pp. 283-298 /1986/

THE ROLE OF DTNB LIGHT CHAIN IN THE CONTRACTILE PROPERTIES OF SKELETAL MUSCLE

Pál Gróf[1], József Belágyi, Árpád Szöőr[x], Sándor Csabina[xx], László Kónya[xx]
Central Laboratory, Medical University, Pécs;
[x]Physiological Institute, Medical University, Debrecen;
[xx]Central Laboratory, Medical University, Debrecen, Hungary

(Received January 31, 1984)

The mechanical and molecular dynamical properties of the glycerinated muscle fibres from m. psoas of rabbit were investigated after treatment with 5,5'-dithiobis/2-nitrobenzoate (DTNB). After DTNB treatment the fibre bundles shortened and exerted force in ATP-relaxing solution. The rate of the ATP hydrolysis in relaxation-inducing medium increased significantly after DTNB treatment, and simultaneously the amount of the LC_2(DTNB) light chain decreased to 40 per cent of the original value measured by gel electrophoresis.

The electron paramagnetic resonance spectra of muscle fibres labelled with maleimide spin label after DTNB treatment exhibited high degree of order of label in rigor, but this order decreased after shortening.

The experiments support the assumption that, in vertebrate skeletal muscles, besides the troponin-tropomyosin regulation system which developed later in evolution, the LC_2 light chain should maintain some regulatory properties.

Introduction

In the theory of sliding filament model which is frequently used to interpret the mechanical events of the muscle function, the motion of myosin heads is assumed (Huxley, 1957, 1969; Harrington, 1979). In experiments using X-ray diffraction technique molecular rearrangements were observed in the internal structure of the thin filaments and in the steric orientation of the heavy meromyosin subfragments, S-1 and S-2 (Miller, Tregear, 1970; Armitage et al., 1975; Seymour, O'Brien, 1980). The rotational motion of myosin heads and the changes in their order could be followed by electron paramagnetic resonance (EPR) spectroscopy, as well (Gróf et al., 1979). The conventional EPR measurements supported

[1]Present address: Institute of Biophysics, Semmelweis Medical University, Budapest

the fact that the myosin heads are arranged on a helical structure along the long axis of the muscle, and the correlation time of their rotational motion is longer than 10^{-7}s, therefore the description of their motional dynamics can be attempted only with the distribution function of spin labels for ordered structures in glycerinated muscle fibres (Gróf, 1977; Belágyi et al., 1980).

One important condition for the myosin ATPase activity is that at least one of the reactive -SH groups (SH 1 and SH 2) or the myosin heads should be unmodified (Sekine et al., 1962; Sekine, Kielley, 1964; Yamaguchi, Sekine, 1966; Bailin, Bárány, 1972; Reisler et al., 1974). The EPR spectra of spin-labelled muscle fibre reflected the highly organized muscle structure only in the case when the paramagnetic probe molecules selectively attached to one of the fast reacting thiol sites (Belágyi, et al., 1979; Thomas, Cooke, 1980). In recent experiments the probe molecules reacted with the SH 2 sites of myosin. The fast-reacting -SH groups - among them the SH 1 groups - had been treated previously with 5,5'-dithiobis(2-nitrobenzoate) (DTNB) which is known to be a reversible -SH inhibitor.

After reactivation of the SH 1 groups following the DTNB-treatment it was found that the fibres shortened under isotonic condition in relaxing solution (in the absence of Ca^{2+}), in contrast to the normal fibres (Gróf et al., 1979). The rate of ATP hydrolysis increased significantly in relaxation-inducing solution and it was the same as in the case of untreated fibres. The aim of the present work was to study the role of the LC_2 (DTNB) light chain in the regulation of the contraction process applying mechanical, biochemical and molecular dynamical (EPR) methods and measurements.

MATERIALS AND METHODS

Investigation of mechanical properties. Glycerinated muscle fibres were prepared from the psoas muscle of rabbit (New-Zealand) weighing 2.5-3 kg according to the method of Szöőr et al. (1981). The excised fibre bundles were fixed on sticks keeping their lengths the same as they were in the body and the membranes of the fibres were destroyed by osmotic shocks. The specimens prepared in such a way could be used already after 48 hours; storing in 50 per cent glycerol at -20°C they keep their

contractile properties for 4-6 weeks. The contraction of the fibre bundles (0.2-0.3 mm diameter and 3-5 mm length) was induced by 5 mM Ca^{2+}-ATP (see Table 1).

Table 1

The composition of solutions used in the investigation of the mechanical properties of glycerinated muscle fibres

solution	EGTA (mM)	ATP (mM)	Ca^{2+} (mM)
A rigor buffer	4	-	-
B R(ATP) relaxing solution	4	5	-
C R(PPi)[x] relaxing solution	4	10[xx]	-
D activating solution	-	5	0.015

The constant components of the solutions are 100 mM KCl, 5 mM $MgCl_2$ and 20 mM TRIS-maleate (pH 7.04).
[x]In R(PPi) solution the KCl concentration was reduced to 80 mM.
[xx]potassium pyrophosphate

The isometric tension was measured with a tension transducer and recorded on an X-t recorder (Szöőr et al., 1981), whereas the isotonic contraction was displayed on a chart recorder using a capacity transducer (Juricskay, 1974).

DTNB-treatment: The glycerol extracted muscle fibres were immersed at first in a solution containing 10 per cent glycerol, 6.67 mM phosphate buffer (pH 7.0) for 15-20 minutes at 4°C; it was followed by washing in solution A (pH 7.0) at 4°C. The composition of the solutions used in the experiments are summarized in Table 1. After washing the glycerol the

fibres were kept for two hours in solution A containing 5 mM DTNB and 5 mM EGTA (pH 7.1, 0°C). The last step of the procedure was a washing again in solution A for 16-24 hours at 4°C. (Gróf et al., 1979).

Spin-labelling: The strategy of the spin-labelling was the modification of the method by Seidel (1969) and Thomas et al. (1975) developed for myosin and extended for muscle in order to ensure the selective labelling (Duke et al., 1976; Reisler et al., 1974) which is necessary for the EPR measurements. The DTNB-treated fibres were kept for two hours in about 20 ml/R(ATP) or R(PPi) relaxing solution containing 0.12-0.25 mM maleimide spin label (pH 7.04, 0°C). The spin-labelling was followed with washing in solution A for 16-20 hours and, thereafter the fibres were treated in solution A containing 2-merceptoethanol in 100-500-fold molar excess over myosin (Gróf et al., 1979). The myosin content was determined according to Arate and Shimizu (1981). Spectrophotometric measurements of the released p-nitrothiophenate ions showed after 2-mercaptoethanol treatment that the number of -SH groups blocked by DTNB was (8.0 ± 0.4) mol/mol myosin, whereas the number of the spin-labelled -SH groups was (0.4 ± 0.1) mol/mol myosin. The number of the spin labelled -SH groups was determined by a comparison of the spectra of denatured muscle samples and that of solution of maleimide spin label with known concentration.

In the experiments where the mechanical properties of the muscle fibre were investigated the fibre bundles were treated in the same way as above, only the maleimide spin label was replaced with N-ethyl-maleimide.

ATPase activity: The ATPase activity was determined by two methods using either the luciferin-luciferase enzyme system or radioactive ATP, ^{32}P isotope in γ-position. In order to enhance the relative amount of the ATP depleted during hydrolysis, the concentration of ATP both in the relaxing and the activating solution was reduced to 1 mM. The fibres were kept in 1 ml solutions; 10 µl and 2 µl samples were taken from the solution to determine bioluminescence or radioactivity. The aliquots were pipetted into 12 per cent TCA neutralized with TRIS buffer. The PEI plates were dried and thin layer chromatography was carried out in phosphate buffer. The radioactivity of the nucleotides and the anorganic phosphate was determined by scintillation counter (Csabina et al., 1982).

SDS-PAGE-electrophoresis: The DTNB-treated muscle fibres were homogenized with a knife homogenizer in the incubating solution. After centrifugation (800 x g, 15 min) the supernate was poured away, and the precipitate was

homogenized again in fresh DTNB-solution and the sample was centrifuged as above. The precipitate was solubilized using the ratio of 1 ml solubilizer to 0.05 g wet muscle. The electrophoresis was carried out in 12.5 per cent SDS gel (Takács et al., 1981). The densitograms were evaluated with a Kipp and Zonen densitometer.

EPR measurements: EPR spectra were taken with an ERS 220 spectrometer (Center of Scientific Instruments, GDR). Small segments ($\sim$ 5 mm) of muscle fibres were mounted parallel to each other in the tissue cell of Zeiss (GDR). Spectra were measured at 20 mW microwave power and 0.2 mT modulation amplitude (100 kHz), fibre axis was oriented parallel and perpendicular to the laboratory magnetic field. The magnetic field was controlled with a proton magnetic resonance magnetometer (MJ 110 R, Radiopan, Poland).

RESULTS

The EPR spectra of muscle fibres spin-labelled on the myosin SH 2 groups exhibited a high degree of orientation dependence of probe molecules in rigor which is a consequence of the selective spin-labelling. Without DTNB-treatment of the muscle fibres the EPR spectra showed no or only little orientation dependence. When the long axis of the muscle fibres was oriented parallel to the magnetic field ($\underline{H} \parallel \underline{k}$), the spectral lines belonging to the $A_{\perp}$ hyperfine coupling constant were predominant (Fig. 1a), on the other hand, when the long axis of the muscle fibres was perpendicular to the magnetic field, the patterns corresponding to the $A_{\parallel}$ coupling constant were strong.

The analysis of the spectra and their computer simulation showed that the $2p\pi$ molecular orbital of the unpaired electron of the paramagnetic probe molecules bound to myosin is perpendicular rather than parallel to the long axis of the muscle (Belágyi, 1975; Belágyi et al., 1979). Since the rotational correlation time of the probe molecules was much longer than 10^{-6}s, the EPR measurements indicated not only the high degree of order of myosin heads in rigor, but they showed that this order was static on the EPR time scale. This supports the fact suggested by other methods that in rigor (almost) all myosin heads are attached to the thin filaments.

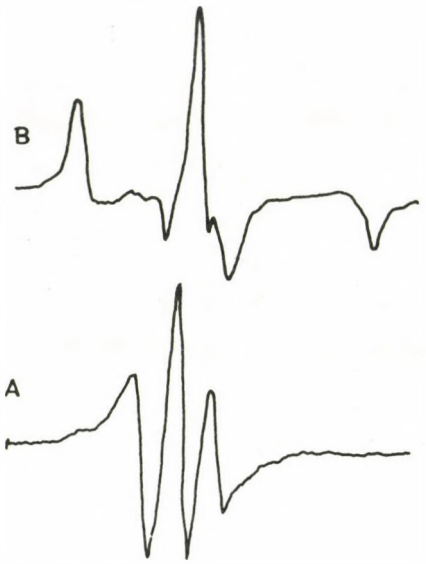


Fig. 1 EPR spectra of spin-labelled glycerinated muscle fibres. After DTNB-treatment the fibre bundles were labelled with maleimide spin label in R(PPi) solution for 30 minutes at 4°C and were treated with 2-MET for 60 minutes (pH 7.4).
A: the long axis of the muscle fibre is oriented parallel with the magnetic field ($\underline{H} \parallel \underline{k}$)
B: the long axis of the fibres is perpendicular to the magnetic field ($\underline{H} \perp \underline{k}$).

In the EPR spectra of DTNB-treated fibres considerable change could be observed at $\underline{H} \parallel \underline{k}$ orientation when the fibres shortened in ATP-relaxing solution. The patterns characterizing the rigor state altered considerably, and the line shape approached that obtained in the case of homogenized muscle fibres (Figs. 2a, b, c).

According to the sliding filament model a part of the myosin heads is detached from the thin filaments in activated state and executes rotational motion. This appears in the EPR spectrum as a decrease of static ordering on the conventional EPR time scale. Thus, the line shape of the EPR spectrum is similar to that measured in the case of randomly oriented probe molecules (homogenized fibre bundles).

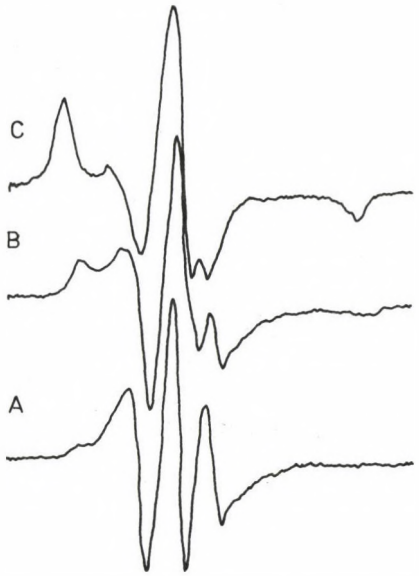


Fig. 2 Conventional EPR spectra of spin-labelled glycerinated fibre bundles in different states of the muscle:
A: rigor
B: activated state
(The long axis of the fibres was oriented parallel to the magnetic field.)
C: homogenized muscle fibres.

In Fig. 3 the mechanical properties of control and DTNB-treated fibres are illustrated under isotonic conditions. The characteristic alteration appeared in ATP-relaxing solution, whereas in the case of pyrophosphate-relaxing solution the mechanical properties of muscles did not differ from that of untreated muscle fibres. At constant length (isometric condition) similar change could be observed in force generation (Figs. 4 a and 4 b).

The ATPase activity of the muscle fibres was measured under isotonic conditions. To minimize experimental error the comparison of the control and DTNB-treated fibres was carried out in parallel measurements. The concentration of ATP in the solutions was reduced to 1 mM. The rate of ATP hydrolysis changed in non-linear manner at longer times, therefore the ATPase activity of the fibres was calculated from the first six

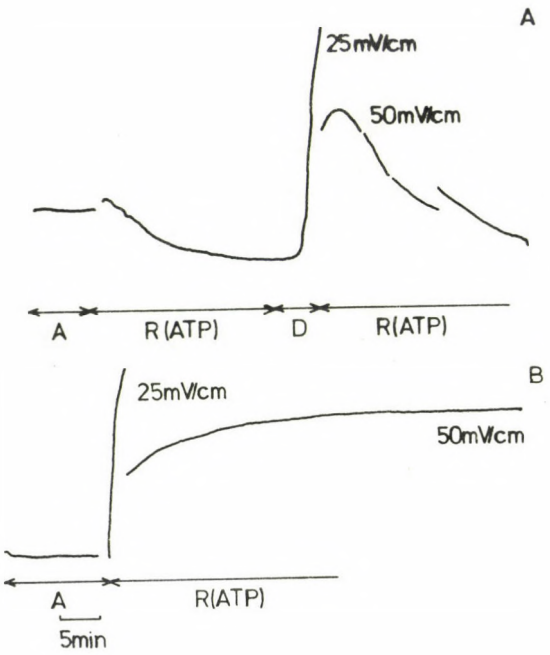


Fig. 3 Mechanical properties of glycerinated muscle fibres under isotonic conditions.
A: control fibres
B: after DTNB-treatment

minutes determination which could be still approximated by linear extrapolation. Fig. 5 shows the rate of ATP hydrolysis of DTNB-treated (D) fibres measured in relaxing solution. The rate of ATPase activity of DTNB-treated and control fibres (N) was D/N = 2.25 in relaxing solution, whereas in activating solution this ratio was 1.05. The values are the mean of six and seven experiments in relaxing and activating solutions, respectively.

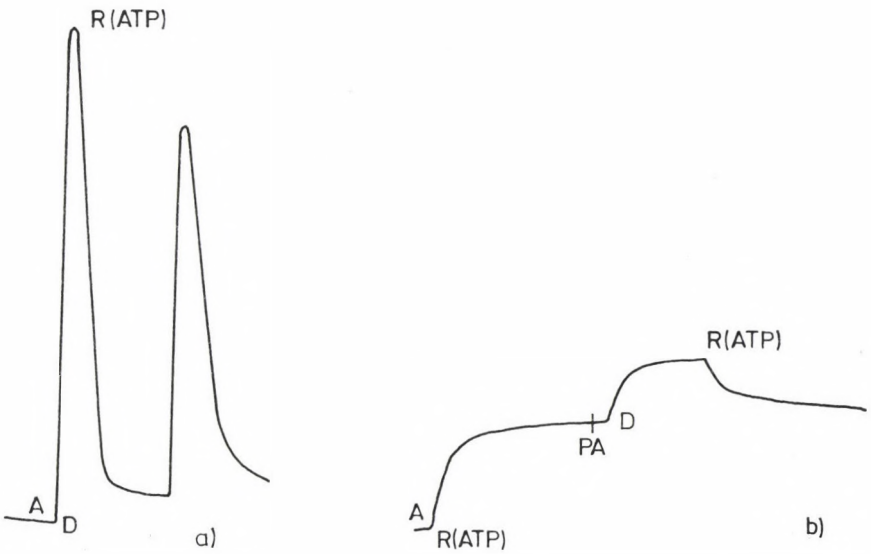


Fig. 4 Mechanical properties of glycerinated muscle fibres from psoas under isometric conditions
left: control fibres
right: DTNB-treated fibres

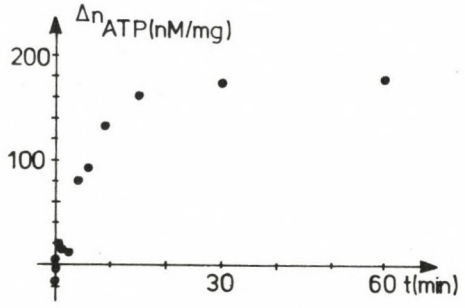


Fig. 5 The rate of ATP hydrolysis of glycerinated muscle fibres isolated from psoas muscles in relaxing solution. The values are normalized for the myosin content of the fibres.

DISCUSSION

According to the theories on the function of vertebrate skeletal muscle the regulation is obtained through the troponin-tropomyosin system. Concerning its molecular mechanism two hypotheses have been developed. Hanson and co-workers (1973) and Oosawa and co-workers (1973) suggested that the regulation in the ATPase activity of myosin is carried out by a change in the conformation of the actin helix. In the second assumption (Haselgrove, 1973; Murray et al., 1980 a, b) the crucial point of the regulation is a competitive reaction between tropomyosin and myosin for the actin binding sites. It was also stated for skeletal muscle of vertebrates that the hydrolysis of LC_2-deficient myosin and actomyosin threads prepared from LC_2-deficient myosin and regulated actin did not change (Weeds, Lowey, 1971; Shrivastava et al., 1980 a, b).
Recent experiment results show that the dissociation of the LC_2 light chain, similarly to natural actomyosin (Csabina, 1982), suppresses the sensitivity of myosin to Ca^{2+}, the fibre bundles turn into a desenzitized state. After DTNB-treatment the SH 1 groups could be reactivated with 2-mercaptoethanol; it shows that the change in the mechanical properties of the muscle is mainly due to the dissociation of the LC_2 light chain.

Based on the results of experiments on actomyosin, Pemrich (1977) pointed out, that the LC_2 light chain stabilizes a particular conformation of myosin and enhances its sensitivity to Ca^{2+}. Thus, the changes in the actin-activated ATPase activity may be attributed to the dissociation of the LC_2 light chain, and not to the modification of the SH groups.

Under isometric conditions, when the maximum tension was achieved in ATP-relaxing solution, further force generation could be induced in activating solution (solution D). After contraction the tension returned to the former level, when the muscle fibres were immersed again in ATP-relaxing solution. Further, but not complete relaxation could be reached with PPi-relaxing solution. Since after the DTNB treatment the LC_2 content of myosin was reduced only to 60 per cent of the original LC_2 content, moreover, the intact cross-bridges did not generate force in ATP-relaxing solution, it is concluded that the maximum tension in ATP relaxing solution is always smaller than the maximum isometric tension. In control fibres the forcer generation of cross-bridges can be stopped

by bringing the fibres either in ATP- or in PPi-relaxing solution. But, on the other hand, in the case of LC_2-deficient cross-bridges, the relaxation of fibres can be achieved only in R(PPi) solution.

In R(ATP) solution the electron micrograms of the muscle fibres treated with DTNB showed a structure characteristic of the isotonic contraction (Fig. 6). The conventional EPR spectra of fibres reflected the high degree of order of probe molecules in rigor; this ordering changes towards randomization in isotonic contraction. The change in the degree of order was significantly smaller in isometric contraction (Gróf et al., 1979) which supports the assumption that the decrease in the order of probe molecules originated at least partly from the desorganization of myofibrils during isotonic contraction.

It is known from previous studies that the rotational state of myosin heads changes during ATP hydrolysis and force generation both in isotonic and isometric contractions (Huxley, 1957, 1969). Nevertheless, it follows from the EPR measurements that the demobilization of the probe molecules did not change in the interaction of myosin with actin, therefore the EPR spectra reflect both the orientation and the rotational state of the myosin head regions. However, there are no direct experimental evidences at present which would prove the motion of myosin heads in muscle fibres in the sub-millisecond time scale, therefore, any conclusion can be drawn about the motion of myosin heads only from the conventional EPR spectra taking into account the degree of ordering which reflects the spatial distribution of the probe molecules. The EPR (Cook et al., 1982) and the optical (Burghardt et al., 1983) measurements showed that during muscle contraction the motional state of a subpopulation of myosin heads was identical with the motional state of heads in rigor, this population corresponds to the myosin heads attached to the thin filaments, whereas the other populations represent the heads in detached state. But it should be noted that during muscle activity changes in the macroscopic ordering cannot be excluded which also appears in the EPR spectrum.

Significant differences were found between the myosin LC_2 light chain contents of DTNB-treated and control fibres in psoas muscle (Fig. 7). The ratios of light chains normalized for the total light chain content were as follows $LC_1:LC_2:LC_3$ = $(0.42 \pm 0.04) : (0.35 \pm 0.03) : (0.25 \pm 0.02)$ and $(0.50 \pm 0.05) : (0.24 \pm 0.03) : (0.25 \pm 0.02)$ for the

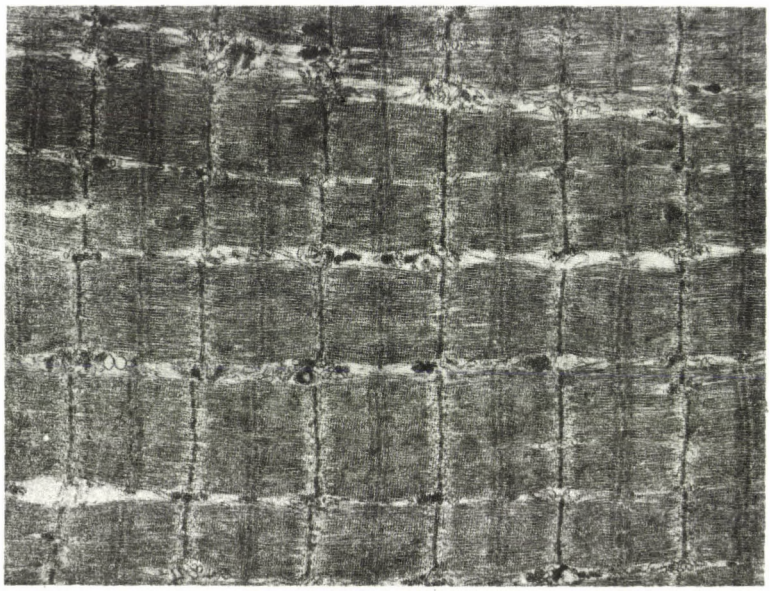


Fig. 6 Electron microgram of DTNB-treated muscle fibres in R(ATP) solution.
Magnification: x 6.000

control and DTNB-treated fibres, respectively. If the decrease of the LC_2-content was normalized for the LC_1- and LC_3-contents than the change was 40 per cent. This result is in good agreement with former measurements on myosin (Weeds, Lowey, 1971; Holt, Lowey, 1975 a, b) and with the results obtained on skinned fibres (Moss et al., 1983)

According to the gel electrophoresis measurements the amount of the troponin-T and troponin-I components remained the same after DTNB treatment, but the amount of the troponin-C was slightly reduced (Fig.7). Since the peaks from the proteins troponin-C and LC_2-light chain partially overlapped in the densitogram, the amount of troponin-C could not be estimated exactly. Nevertheless, a decrease of the amount of troponin-C would enhance the inhibitory effect which prevents shortening or force generation in a Ca^{2+}-free medium. However, our experiments do not exclude the possibility that the DTNB-treatment induces conformational changes in the troponin system which lead to the alteration of the mechanical properties of the muscle fibres.

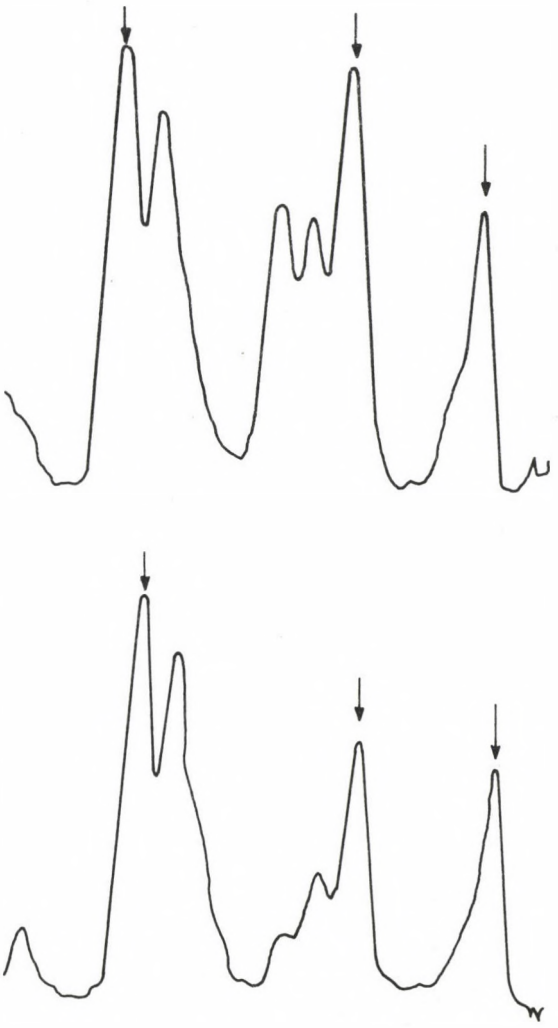


Fig. 7 Densitograms of the SDS PAG electrophoreses of glycerinated muscle fibres
upper: control fibres
bottom: DTNB-treated fibres.
The arrows from left to right denote the LC_1, LC_2 and LC_3 light chains.

Moss et al. (1982, 1983) reported that the removal of the LC_2 light chain was accompanied by a decrease of the maximum velocity of shortening and, simultaneously, the maximum isometric tension was also slightly reduced. It was also pointed out that the changes could be specifically attributed to the removal of the LC_2 light chain and not to the alteration in the amount of troponin-C component accompanying the treatment.

The experimental findings suggest that in the skeletal muscle of vertebrates the LC_2 light chain would maintain some regulatory property linked to myosin, besides the troponin-tropomyosin regulatory system which developed later in the evolution. The removal of the LC_2 light chain induce conformational changes in the head region of myosin which prevent the regulation of the troponin-tropomyosin system in the actin-myosin interaction. This assumption would mean that the myosin-linked regulation observed mainly in the muscles of lower order living organism would exist to some extent in the muscles of higher order organisms, as well.

Acknowledgement

This work is a part of the research project, Mechanism of Regulation in Life Processes and was supported by a grant of the Ministry of Health. The authors thank Dr. K. Hideg (Central Laboratory, Medical University, Pécs) for supplying the maleimide spin label and Dr. Ö. Takács (Institute of Biochemistry, Medical University, Szeged) for evaluation of the gel electrophoresis experiments.

REFERENCES

Arata, T., Shimizu, H. (1981) J. Mol. Biol. 151 411-437

Armitage, P.M., Tregear, R.T., Miller, A. (1975) J. Mol. Biol. 92 39-53

Bailin, G., Bárány, M. (1972) J. Biol. Chem. 247 7815-7821

Belágyi, J. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. 10 233-242

Belágyi, J., Schwarz, D.D., Damerau, W. (1979) Studia Biophys. 77 77-83

Belágyi, J., Gróf, P., Pallai, G. (1980) Acta Biochim. Biophys. Acad. Sci. Hung. 15 81-88

Burghardt, T.P., Ando, T., Borejdo, J. (1983) Proc. Nat. Acad. Sci. USA 80 7515-7519

Cooke, R., Crowder, M.S., Thomas, D.D. (1982) Nature 300 776-778

Csabina, S., Csongor, J., Kónya, L., Szöőr, Á. (1982) Kísérletes Orvostud. 34 308-312

Csabina, S. (1982) Acta Physiol. Acad. Sci. Hung. (in press)

Duke, J.R., Takashi, K.U., Morales, M.F. (1976) Proc. Nat. Acad. Sci. USA 73 302-306

Gróf, P. (1977) (competition paper)

Gróf, P., Belágyi, J., Pallai, G. (1979) "Symposium on Stable Nitroxide Free Radicals; Synthesis and Application" Pécs, 1979

Hanson, J., Lednev, V., O'Brien, E.J., Bennett, P.M. (1973) Cold Spring Harbor Symp. Quant. Biol. 37 311-318

Harrington, W.F. (1979) Proc. Natl. Acad. Sci. USA 76 5066-5077

Haselgrove, J.C. (1973) Cold Spring Harbor Symp. Quant. Biol. 37 341-352

Holt, J.C., Lowey, S. (1975 a) Biochemistry 14 4600-4609

Holt, J.C., Lowey, S. (1975 b) Biochemistry 14 4610-4620

Huxley, A.F. (1957) Prog. Biophys. Chem 7 255-318

Huxley, H.E. (1969) Science 164 1356-1366

Juricskay, S. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. 9 151-158

Miller, A., Tregear, R.T. (1970) Nature (London) 226 1060-1061

Moss, R.L., Giulian, G.G., Greaser, M.L. (1982) J. Biol. Chem. 257 8588-8591

Moss, R.L., Giulian, G.G., Greaser, M.L. (1983) J. Cell Biol. 96 970-978

Murray, J.M., Knox, M.K., Trueblood, C.E., Weber, A. (1980 a) FEBS Letter 114 169-173

Murray, J.M., Weber, A., Wegner, A. (1980 b) In "Muscle Contraction: Its Regulatory Mechanism (Ebashi, S. et al., eds.) pp. 221-236 Springer-Verlag, Berlin

Oosawa, F., Fujima, S., Ishwata, S., Mihashi, K. (1973) Cold Spring Harbor Symp. Quant. Biol. 37 277-286

Pemrick, S.M. (1977) Biochemistry 16 4047-4057

Reisler, E., Burke, M., Harrington, W.F. (1974) Biochemistry 13 2014-2022

Seidel, J.C. (1969) Biochim. Biophys. Acta 180 216-219

Sekine, T., Barnett, L.M., Kielley, W.W. (1962) J. Biol. Chem. 237 2769-2776

Sekine, T., Kielley, W.W. (1964) Biochim. Biophys. Acta 81 336-340

Seymour, J., O'Brien, E.J. (1980) Nature 283 680-682

Shrivastava, S., Cooke, R., Wikman-Coffelt, J. (1980) Biochem. Biophys. Res. Commun. 92 1-7

Shrivastava, S., Wikman-Coffelt, J. (1980) Biochem. Biophys. Res. Commun. 92 1383-1388

Szöőr, Á., Rapcsák, M., Boross, A. (1981) Acta Biochim. Biophys. Acad. Sci. Hung. 16 101-103

Takács, Ö., Szöőr, Á., Sohár, I., Kesztyüs, L., Guba, F. (1981) Acta Biol. Acad. Sci. 32 33-43

Thomas, D.D., Seidel, J.C., Gergely, J., Hyde, J.S. (1975) J. Supramol. Struct. 3 376-390

Thomas, D.D., Cooke, R. (1980) Biophys. J. 32 891-906

Weeds, A.G., Lowey, S. (1971) J. Mol. Biol. 61 701-725

Yamaguchi, M., Sekine, T. (1966) J. Biochem. (Tokyo) 59 24-33

Book Reviews

Diagnosestrategien by D. Scheuch, R.J. Haschen, L. Neef (eds.) (Ergebnisse der Experimentellen Medizin, 43 VEB Volk und Gesundheit, Berlin, 1983, 245 pp.

The volume contains the lectures of the symposium "Diagnostic strategies" held in Halle between 24-26 September, 1981. The meeting was organized by the Martin Luther University, Halle-Wittenberg and the Clinical Chemical and Laboratory Diagnostical Society of the GDR. Its aim was to help the colleagues working in clinical practice to develop more efficient diagnostic strategies for the more optimal selection of the parameters and for the more objective evaluation of the findings. Besides the new results of the clinical biochemical and laboratory diagnostics, the modern multivariational statistical procedures have been presented in 10 sections during the Symposium. Accordingly, the volume comprises 10 chapters.

In Chapter 1 in the general topics three lectures were held. The first one (problem-oriented diagnostics in the practice of internal medicine) gives numerous useful advices mainly to the young clinical physicians summarizing the error possibilities and the most frequent difficulties of diagnoses made on the basis of symptoms and syndromes. Citing American experiments it emphasizes the importance of the close cooperation with the physicians making laboratory and X-ray examinations in the diagnostical work. Finally it analyzes and advocates the usage of the so called Weed-type "problem oriented case sheet writing" in a wider range. Of the following two lectures the one dealing with the teaching of the applicability of clinical biochemistry excels.

Chapter 2 describes the more recent multivariational statistical procedures and the possibilities of their application e.g. in ambulant diagnostics and in the distinction of gastrointestinal hemorrhages.

Chapter 3 discusses two lectures. The one titled "The importance of laboratory diagnostics in intensive medical care" summarizes the groups of diseases in which the close control of laboratory examinations within the frame of intensive care is of utmost necessity and enumerates the clinical biochemical parameters it proposes either as fundamental or as

supplementary examinations, respectively. The lecture titled "The algorhythms in the study of the disorders in the acid-base equilibrium" contains similarly a lot of useful information important in everyday medical practice as well.

Chapter 4 deals with liver diseases. The lecture titled "The diagnostic significance of serum protein concentrations in liver diseases" discusses the albumin, prealbumin, cholinesterase, prothrombin, haptoglobin, coeruloplasmin, alpha-1 antitrypsin, alpha-2 macroglobulin, orosomucoid and transferrin levels and their variations in different hepatopathies, as compared with a healthy control group. Further on, two more lectures deal with the significance of the alanine-amino-peptidase isoenzyms, the GOT, ALP and LDH isoenzymes in liver and gall-bladder diseases, specially emphasizing the reconvalescent stage of hepatitis. The last paper analyzes the serum bile acids with Silufol plate method.

Chapter 5 outlines the diagnostic strategy of acute pancreatitis and draws special attention to the numerous other diseases presenting differential diagnostic problems, in which the serum amylase, and in some cases the lipase too, may increase.

In Chapter 6, among the metabolic diseases is to be found the lecture of the sole Hungarian author, L. Vértes, containing many ideas to be taken seriously on the difficulties of the diagnosis of the old-age diabetes mellitus. The lecture with the title "The significance of apolipoproteins in the diagnosis of the disorders in the lipoprotein metabolism" treats in detail the AI, AII, B, CI, CII, CIII, D and E apolipoproteins.

The subject of Chapter 7 is nephrology. The lecture describing briefly the parameters, their limiting values and methods of examination in the diagnosis of nephropathies contains questionnarie-type printed forms as well, which are mainly of pediatric orientation. The next lecture proposes the calculation of the amylase-creatinine clearance ratio for the determination of the function of proximal tubules. If this method proves to be good in clinical practice, it may replace the difficult and expensive beta-2 microglobulin and lysozime determination used up to now to measure the function of the proximal tubules. Further two lectures deal with the insulin and PAH clearances and with the recognition of patients having Ca-oxalate renal stone.

Chapter 8 deals with heart and circulatory diseases. The subject of three out of four lectures is about shock. One lecture proposes the determination and follow-up of the so called indicator enzymes (LDH, ASAT, ALAT, GLDH, ICDH, MDH and CK) for the determination and control of the shock syndromes of various origin, a further one analyzes the significance of kallikrein in experimentally produced and clinical shock.

The single lecture of Chapter 9 analyzes the biochemical parameters of the diagnosis of hypoxia and Chapter 10 briefly summarizes the ratios and quantity of the protein-type and cell-like components of the cerebrospinal fluid and their relations in multiple sclerosis.

From the wide-ranging topics of the Symposium both the physician colleagues working in clinical practice and those in the chemical laboratories may obtain useful information.

Judit Nagy

Electron Beam Interactions with Solids for Microscopy, Microanalysis and Microlithography by D.F. Kyser, H. Niedrig, D.E. Newbury and R. Shimizu (eds.) Scanning Electron Microscopy, Inc., Chicago (USA), 1984.

This book was published by the Scanning Electron Microscopy, Inc. in 1984. What this book deals with, is well reflected by its long title. But if we want to characterize it in short then we have to point out that it is an excellently edited, high level work.

As a matter of fact, it contains the materials of the 1st Pfefferkorn Conference, held April 18 to 23, 1982, a the Asilomar Conference Center, Monterey, USA. The edition of the volume started with the invitation of distinguished scientists as lecturers. They can be seen on the VI page of the book and those readers, who are familiar with this field, wonder how the organizers succeeded in collecting so many xcellent scientists for this event.

The title of the book suggests that this collection of papers may be of interest for those who are engated in electron microscopy, electron

beam microanalysis or in the microlithography which is of importance in the semiconductor technology. By this short book review I should like to draw the attention that this book may be interesting for others as well.

Arbitrarily, I divide the book into four parts. The first part which is about the inelastic scattering of electrons may be a real treat for theoreticians too, who are not engaged in electron microscopy. Into this first part I put the paper by M. Inokuti and S.T. Manson as well as that by C.J. Powell. Both papers are suitable for the purpose of education at universities.

To the second part I would count such papers which are theoretical but their connection with the practical work is so close that the microscopist or microanalyst meets these problems every day. I should like to mention only some of these papers which have the common feature that they give an overall picture of a particular field: on the secondary electron emission by H. Seiler, on the backscattering by H. Niedrig, on the energy distribution of excitation by D.B. Wittry and on the electron--matter interaction in the scanning microscopy wrote by K. Kanaya and S. Ono. (In this part on page 66. I have found the only citation of a Hungarian work: Z. Bödy, 1962, On the Backscattering of Electrons from Solids, Brit. J. Appl. Phys. 13 483-485).

The third part of the volume is linked with the mathematical method applied, i.e. the Monte Carlo technique. Although the mathematical method is common the topics are still rather manifold. The paper of D.F. Kyser has no be mentioned from that point of view that it may serve as a good starting point for those who are interested in the Monte Carlo technique but are not yet familiar with it. A long list of references with items containing ready computer programs aims at helping the beginners, having access to aa larger computer, to start on the rough road of Monte Carlo. (In a personal discussion with one of the conference participants Professor Murate, it has turned out that for a detailed simulation of the electron beam interaction with targets a 64 kbyte Apple computer is too small and, above all, too slow.)

The Monte Carlo technique is of great importance in the electron beam microanalysis because it makes possible to calculate the electron backscattering and the X-ray excitation (papers by E.D. Newbury, R.L. Myklebust and J.D. Brown). Furthermore, it plays a role in the EBIC and

cathodoluminescence analyses, due to the possibility of calculating the so-called depth-dose and lateral-dose functions (S.P.Shea's paper). But I could go on with citing the Auger analysis (R. Shimizu and S, Ichimura) or thin film microanalysis (K. Murate) in which a good use can be made of the Monte Carlo technique.

Into the fourth part of the book I count some methodical papers. L. Reimer has written on the detection of electrons and on the detector strategy. Some papers deal with the electron energy loss spectroscopy (EELS). This is the most fashionable method nowadays. From R. Leapman's paper we can learn what information can be obtained about solids by means of EELS and this review paper gives much what should be known about ELLS. The theoretical aspects of the same method (serial and parallel detection) is discussed by D.C. Joy.

The publisher, the Scanning Electron Microscopy, Inc. has organized a strict reviewing system which ensures that only papers of high quality are published. I suggest this book for everybody who is working in the field of microanalysis and electron microscopy or Auger analysis and do not want to be only an operator of a particular equipment. Furthermore, I recommend this book as manual to university lecturers.

I. Pozsgai

Stopping Powers for Electrons and Positrons; ICRU Report 37; International Commission on Radiation Units and Measurements, Bethesda, MD. 20814 USA.

The discovery of ionizing radiations in close connection with subsequent further discoveries of great importrance resulted in the development of radiology, a multi-disciplinary new branch of science. Well ahead of other branches, medical science was the first in the world to detect the practical importance of radiology and to make use of it in its arsenal already at the end of the last century. In the course of introducing methodologies that opened up earlier unimaginable new

horizons to medical diagnostics and therapy, the importance of exact dosage of different photon and corpuscular radiations became more and more obvious, as well as the importance of reliable, uniform measurements for the purpose of radiation protection.

After early attempts of three decades, in 1925 the first National Radiological Congress called a committee named International Commission of Radiological Units and Measurements (ICRU) into being, in order to work out internationally acceptable proposals

- on concepts denoting the quantity of ionizing radiations and radioactive substances and on the methods of their measuring in clinical radiology and radiobiology, furthermore

- on the numerical values of those fundamental physical quantities which can secure the homogeneity and comparability of the measured data in the international publications.

ICRU, which has already been working for 60 years, circumspectly performs its important task serving the exact dosage measurement of ionizing radiations harmonizing its work and proposals with the recommendations of ICRP (which was also established in 1925) and with several other international organizations, like WHO, ILO, UNSCEAR, ISO, etc.

Since medical radiology develops rapidly newer and newer demands on dosimetry are coming up. In addition to these, the application of ionizing radiations is extended to more and more territories of everyday life. Moreover radiology, in consequence of the radiation protecting aspects, begins to become one of the basic questions of manking. Therefore, ICRU regularly supervises its recommendations, reworks and renews them in accordance with the increase of our knowledge in radiation physics and radiobiolgy.

At present, ICRU works with 15 commissions on compiling different reports. One of these reports is Report 37 entitled: Stopping Powers for Electrons and Positrons. It was issued in 1984 and was worked out by a scientific commission of 14 members working under the chairmanship of H.O. Wyckoff. The Report consists of an introductory part of 8 pages and of the actual work of 271 pages.

The extremely valuable summary of our theoretical knowledge about making the tables is contained in the first part of the actual work on 57 pages

which was compiled by the editors closely following the gradual development of the past decades on the basis of the special literature of theoretical physics.

The titles of the most important chapters: 2. Formulae for the Collision Stopping Power, - 3. Methods for Estimating Mean Excitation Energies, - 7. Restricted Collision Stopping Power, - 9. Radiative Stopping Power, - 11. Miscellaneous Comparisons. We come to know that the first tables of this kind were calculated and presented by NELMS in 1956. They were followed by newer and newer tables taking the gradual development of the theory into consideration. The report surveys and analyses the agreements or deviations between calculations and measurements, then determines those eight new aspects - among them the first used differentation for electrons and positrons in regard to the radiative stopping power - which make the tables particularly valuable.

The second part with 202 pages contains the main value of he report, the tables including about 100.000 data altogether. In these the Stopping Powers, Rangers and Radiation Yiedls can be found and some other data with respect to electrons for 27 elements and 49 substances that are important from the point of view of dosage measurement, e.g. air, bone, bone-equivalent plastic, muscle, muscle-equivalent liquid, paraffin wax, photographic emulsion, plastic scintillator, etc., and for 6 elements and 17 different substances with respect to positrons.

Finally, the report contains a detailed literary survey of 245 titles and it is closed by an Index.

L. Bozóky

Flavonoids and Bioflavonoids. Proceedings of the 7th Hungarian Bioflavonoid Symposium. (Edited by L. Farkas, M. Gábor and F. Kállay). Joint edition of Akadémiai Kiadó and Elsevier Science Publishers, Budapest (Hungary), 1986. XXII+466 pp. 17 x 24.5 cm. ISBN 963054313 3.

This photooffset volume is an outgrowth of the Seventh Hungarian Bioflavonoid Symposium, held May 16-18, Szeged, Hungary, with participants from 15 countries, under the sponsorship of the Hungarian Academy of Sciences and several

pharmaceutical and chemical firms in Hungary. Similarly to the predecessor volumes of these periodically organized Symposia, this book contains a collection of 44 lectures, dealing with various contemporary aspects of this intensely active field of heterocyclic natural products chemistry and biochemistry.

The major subjects of the volume cover the widest possible fields of flavonoid research in a broad interdisciplinary approach. The reader can find up-to-date information about the newly developed isolation techniques of flavonoids and related compounds, as well as about the structural and stereochemical investigation of such naturally occuring heterocyclic substances. Some of the lectures discuss novel strategies and reagents useful for the chemical synthesis and conversion of flavonoid compounds. Another contributions deal with the pharmacological properties and mode of action of biologically active flavonoids, as well as with the possible application thereof as new medicinal agents.

Quantum chemical studies, performed with flavonoid compounds, may contribute to an ultimate exploration of the chemical structure-biological activity relationship of this family of organic compounds, and modern separation techniques, presented in a few lectures, can help in the identification of the effective components and metabolites of various flavonoid-containing natural substances, being potential sources of pharmaceutical preparations in the future. On the other hand, such experience might be also useful in additional fields of natural products research.

As the results included in the volume involve the fields of organic chemistry, biochemistry, phytochemistry, plant physiology and taxonomy, pharmacology and medicinal practice, this collection of papers appears to be extremely useful for chemists, botanists, pharmacologists and medical doctors engaged in the research and practical application of flavonoids and related organic compounds.

I. PELYVÁS

A desirable plan for the organization of a paper is the following: Summary, Introduction, Materials and methods, Results, Discussion, References.

A clear and informative title is very important. Provide a short title (not to exceed 50 characters and spaces) to be used as a running head. Every paper must begin with a **Summary** (up to 200 words) presenting the important and pertinent facts described in the paper.

The **Introduction** should state the purpose of the investigation, but should not include an extensive review of the literature. The description of the **Materials and methods** should be brief, but adequate for repetition of the work. Refer to previously published procedures employed in the work. It is strongly recommended that author(s) should draw attention to any particular chemical or biological *hazards* that may occur in carrying out experiments described in the paper. Relevant safety precautions should be suggested. The **Results** may be presented in tables or figures. The **Discussion** should be concise and deal with the interpretation of the results. In some cases combining **Results and discussion** in a single section may give a clearer presentation.

References to the literature in the text should be by numbers in parentheses. In the reference list the items should be arranged in order of these serial numbers. For citation note the following examples:

1. Drust, D. S. and Martin, T. F. J. (1985) Biochem. Biophys. Res. Commun. 128, 531–537
2. Hoyer, S. (1980) in Biochemistry of Dementia (Roberts, P. J. ed.) pp. 252–257, J. Wiley and Sons, New York

Abbreviations and symbols defined in the IUPAC-IUB Document No. 1 (Arch. Biochem, Biophys. *115*, 1–12, 1966) may be used without definition, but others are to be avoided. When necessary, *abbreviations must be defined in a footnote and typed single-spaced on a separate page*, rather than in the text. Abbreviations *must not* be used in the Summary. Enzyme names should not be abbreviated except when the substrate has an accepted abbreviation (e.g. ATPase). Use of Enzyme Commission (EC) code number is required when available. Styling of isotopes should follow the recommendations of the IUB Commission of Editors. The symbol of the isotope should be placed in brackets attached directly to the front of the name, e.g. [^{32}P] AMP. Isotope number should only be used as a (superior) prefix to the atomic symbol, not to an abbreviation.

Typing of manuscript

The accepted articles are reproduced directly from the submitted manuscript as camera ready copy, thus *no changes can be made in the manuscript after acceptance*. Therefore black silk or carbon typewriter ribbon should be used. For obvious reasons proofs are not required and cannot be supplied. Special care should be taken to ensure a sharp, clean impression of letters throughout the whole manuscript. It is strongly recommended to use an *electric typewriter*. Erasures or other corrections should be avoided. Spelling, word division and punctuation must be carefully controlled.

The title of the paper should be typed in capital letters near the top of the first page, with the name(s) an affiliation(s) of the author(s) just typed below. Manuscripts should be double-spaced; methods, references, abbreviations, footnotes and figure legends should be *single-spaced*. Typing area of the page must be as close as possible to 16 × 24 cm.

Each page should be numbered at the bottom in light blue pencil marks. On a separate sheet indicate a running title, not more than 6 key-words for subject indexing and the author to whom reprints are to be sent, including a telephone number if possible.

Illustrations and tables. Do not attempt to insert figures, figure legends or tables into the text. This will be done by the Publisher. Original drawings should be clearly labelled in black ink in a manner suitable for *direct reproduction*. Typewritten lettering is not acceptable. Figures will be reduced to fit within the area of the page. All letters, numbers and symbols should be drawn to be at least 2.5 mm high after reduction. Glossy photographs or drawings are acceptable provided they are sharp, clear prints with an even black density. Illustrations in colour cannot be accepted. Mathematical and chemical symbols that cannot be typed should be drawn carefully in black. Tables and illustrations should be submitted on separate sheets.

One hundred reprints will be supplied free of charge.

CONTENTS

Index: 26.030

FORMERLY ACTA BIOCHIMICA ET BIOPHYSICA ACADEMIAE SCIENTIARUM HUNGARICAE

Acta Biochimica et Biophysica Hungarica

VOLUME 21, NUMBER 4, 1986

Akadémiai Kiadó, Budapest
ABBPAP 21 (4) 307—399 (1986) HU ISSN 0001-5253

Acta Biochimica et Biophysica Hungarica

a Quarterly of the Hungarian Academy of Sciences

Editors

P. ELŐDI and J. TIGYI

Managing editors

P. GERGELY and A. NIEDETZKY

Acta Biochimica et Biophysica Hungarica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

H-1054 Budapest, Alkotmány u. 21

Subscription information

Orders should be addressed to

KULTURA Foreign Trading Company

H-1389 Budapest P.O.Box 149

or to its representatives abroad.

Acta Biochimica et Biophysica Hungarica is indexed in Current Contents

INSTRUCTION TO AUTHORS

Acta Biochimica et Biophysica Hungarica will primarily publish original and significant papers from Hungarian research institutes, universities and other laboratories. Original papers written in English in diverse fields of modern experimental biology will be considered for publication. Novel aspects of the information described in the paper should be clearly emphasized.

Manuscripts (one original and two copies) should be submitted to

Dr. P. Gergely, Department of Medical Chemistry, University Medical School, 4026 Debrecen, POB 7, Hungary *(Biochemistry)*

or

Dr. A. Nidetzky, Department of Biophysics, University Medical School, 7624 Pécs, POB 99, Hungary *(Biophysics)*.

To ensure rapid and accurate publication, the author(s) are invited to follow the instructions described below. Manuscripts which do not conform these rules will be returned.

Preparation of manuscript

Full-length papers should not exceed 10 typewritten pages on A4 size high quality paper. The complete manuscript including the space occupied by figures, tables, references, acknowledgement etc. should not exceed 15 pages.

Short communications of total length of 5 pages will also be accepted.

Acta Biochim. Biophys. Hung. 21(4), 307-311 (1986)

BILE PIGMENTS INHIBIT MICROSOMAL LIPID PEROXIDATION

István Wolfram, Miklós Végh and István Horváth

Institute of Biochemistry Dept. 2, Semmelweis University Medical School, H-1444 Budapest, Hungary

(Received April 5, 1983)

SUMMARY

Bile pigments (bilirubin and biliverdin) inhibit lipid peroxidation in rat liver microsomes at physiological concentrations. Since an alternative course for bilirubin degradation involving superoxide radical has been described, a possible explanation of the decrease of the initial rate of lipid peroxidation could be the scavenging of $^{\cdot}O_2^-$.

Various free radicals of oxygen are involved in lipid peroxidation. Superoxide is known to play a role in the initiation of the process because its enzymic conversion to peroxide by superoxide dismutase may result in an inhibition of peroxidation (Svingen et al., 1978; Bus and Gibson, 1979). Although lipid peroxidation is thought to start by the attack of hydroxy radicals generated in the iron-catalyzed Haber-Weiss reaction (Fong et al., 1973) at lipid hydrocarbon, evidence has accumulated suggesting alternative mechanisms not depending upon $^{\cdot}OH$ (Tien et al., 1982).

The inconsistency in the literature concerning the role of $^{\cdot}O_2^-$ in the initiation of lipid peroxidation prompted us to investigate the effects of bile pigments as $^{\cdot}O_2^-$ scavengers on the

Correspondence to: Dr. I. Horváth, Institute of Biochemistry Dept. 2, Semmelweis University Medical School, H-1444 Budapest, P.O.B. 262.

Akadémiai Kiadó, Budapest

process. It is known that photodecomposition of bilirubin is substantially increased in the presence of xanthine oxidase generating superoxide. Singlet oxygen-quenching agents or $^{\cdot}OH$ scavengers do not prevent this enhancement of bilirubin oxidation (Kaul et al., 1979), but either the bleaching of bilirubin or that of biliverdin is inhibited by low levels of manganese superoxide dismutase (Robertson and Fridovich, 1982). Moreover, it has been shown that in vivo generation of superoxide radical can stimulate the clearance of bilirubin from plasma in experimental hyperbilirubinemic rats (Kaul et al., 1980). In our experiments bile pigments at physiological concentrations were found to inhibit lipid peroxidation (malondialdehyde formation) in rat liver microsomes.

Rat liver microsomes were isolated from male rats of 170-200 g according to the method of Pederson et al. (1973). Microsomal pellet was stored at -20°C and resuspended in 0.15 M Tris-HCl buffer (pH 7.4) prior to use. Protein concentration in the incubation samples was 0.4 mg/ml as measured by the method of Lowry et al. (1951).

Malondialdehyde production served as an index of lipid peroxidation. Malondialdehyde was measured according to the method of Ottolenghi (1959).

Experiments were carried out at 37°C in a medium consisting of 0.15 M Tris-HCl buffer (pH 7.4). The reaction mixtures contained 0.3 mM NADPH, 15 μM Fe^{3+} chelated to ADP of a final concentration of 3 mM. Bilirubin and biliverdin were freshly dissolved in 0.1 M NaOH and adjusted to the final concentration with 0.15 M Tris-HCl buffer (pH 7.4). Stock solutions of bile pigments and reaction mixtures were stored at 0°C in the dark. After preincubation for 2 minutes the reaction was initiated by adding microsomes to the incubation mixtures. Samples were withdrawn at the times indicated and assayed for malondialdehyde production.

Bilirubin was obtained from Gedeon Richter Ltd. (Hungary) and biliverdin from Sigma Chemical Co., (St. Louis, USA). All other reagents used were of analytical grade.

In the presence of ADP-Fe^{3+} malondialdehyde formation started immediately. Both bilirubin and biliverdin had a significant inhibitory effect on microsomal lipid peroxidation induced by NADPH and Fe^{3+}. This inhibition by bile pigments was concentration dependent and caused a lag phase even at low concentrations (Figs. 1 and 2). At higher concentrations, the initial rate of malondialdehyde production decreased substantially and the lag phase was prolonged. At 10 µM both bile pigments totally inhibited induced microsomal lipid peroxidation.

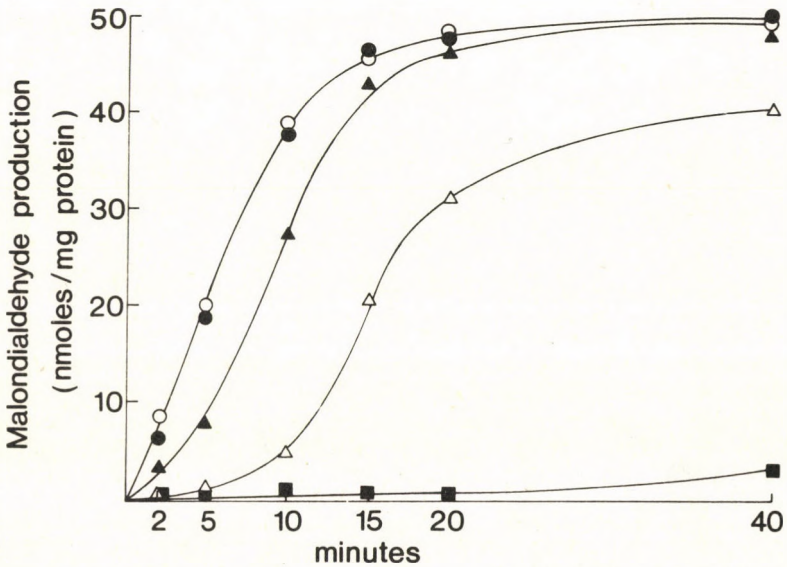


Fig. 1. Effect of bilirubin on the time course of microsomal lipid peroxidation induced by NADPH and ADP-Fe^{3+}. Reaction mixtures contained 0.4 mg/ml microsomal protein in 0.15 M Tris-HCl (pH 7.4) at 37°C. The following additions were made as indicated: (o) ADP-Fe^{3+} + NADPH at concentrations described in Materials and methods; (●) (▲) (△) (■) ADP-Fe^{3+} + NADPH and 1, 2.5, 5 and 10 µM bilirubin, respectively. The results of a typical experiment are shown.

An alternative pathway for bilirubin degradation involves superoxide radical (Kaul et al., 1980) which might explain our observation. It should be noted that the inhibition of micro-

somal lipid peroxidation by bile pigments occurs at non-pathologic concentrations. It is possible that at higher concentrations bilirubin or biliverdin could act as part of a natural defense mechanism against the toxic effects of oxygen.

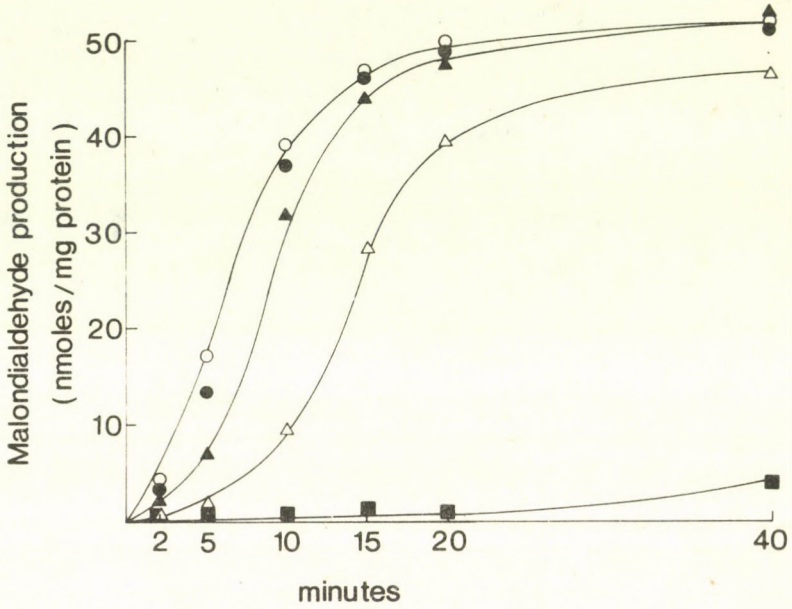


Fig. 2. Effect of biliverdin on the time course of microsomal lipid peroxidation induced by NADPH and ADP-Fe^{3+}. The experiment was carried out as described in the legend of Fig. 1 and biliverdin was added at the same concentrations as bilirubin.

REFERENCES

Bus, J.S. and Gibson, J.R. (1979) in Review in Biochemical Toxicology (Hodgson, E., Bend, J.R. and Philpot, R.M. eds.) pp. 125-149, Elsevier-North Holland, New York

Bonnett, R. (1981) in Essays in Biochemistry (Campbell, P.N. and Marshall, R.D. eds.) pp. 1-51, Academic Press, London

Fong, K., McCay, P.B., Poyer, J.L., Keele, B.B. and Misra, H. (1973) J. Biol. Chem. 248, 7792-7797

Kaul, R., Kaul, H.K., Bajpai, P.C. and Murti, C.R.K. (1979) J. Biosci. (India) 1, 377-383

Kaul, R., Kaul, H.K. and Murti, C.R.K. (1980) FEBS Lett. 111, 240-242

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Ottolenghi, A. (1959) Arch. Biochem. Biophys. 79, 355-363

Pederson, T.C., Buege, J.A. and Aust, S.D. (1973) J. Biol. Chem. 248, 7134-7141

Robertson, Jr. P. and Fridovich, I. (1982) Arch. Biochem. Biophys. 213, 353-357

Svingen, B.A., O'Neal, F.O. and Aust, S.D. (1978) Photochem. Photobiol. 28, 803-809

Tien, M., Svingen, B.A. and Aust, S.D. (1982) Arch. Biochem. Biophys. 216, 142-151

Acta Biochim. Biophys. Hung. 21(4), 313-325 (1986)

POLY(ADP-RIBOSE) AND REPLICATIVE DNA SYNTHESIS STUDIED IN PERMEABLE MOUSE THYMOCYTES

Ágnes Soóki-Tóth, Sándor Csuzi, Hans Altmann[x], Ferenc Antoni and Gáspár Bánfalvi[o]

Institute of Biochemistry Department I. Semmelweis University Medical School, H-1444 Budapest, Hungary and [x]Institute of Biology, Austrian Research Center, A-1082 Vienna, Austria

(Received December 25, 1985)

SUMMARY

Poly(ADP-ribose) and DNA synthesis were followed in a random population of freshly isolated and permeabilized mouse thymocytes. Different conditions were found to be optimal for the synthesis of poly(ADP-ribose) and DNA, respectively. The suspension of one of the processes did not influence significantly the synthesis of the other macromolecule. Our results suggest that there is no direct relationship between the two processes.

INTRODUCTION

Poly(ADP-ribose) synthetase is a nuclear enzyme of eucaryotic cells that is covalently bound to nuclear proteins (Chambou et al., 1966; Hayaishi and Ueda, 1977; Purnell et al., 1980) catalysing the formation of poly(ADP-ribose) from NAD. Although the physiological importance of the synthesis of this homopolymer is not yet fully established, it is thought to be involved in processes related to the alteration of chromatine structure including transcription (Muller et al., 1974; Muller and Zahn, 1976; Furneaux and Pearson, 1978), replication (Burzio and Koide, 1970; Yoshidara and Koide, 1973; Kitamura et al., 1979) and cell differentation (Caplan and Rosenberg, 1975).

Key words: DNA-synthesis; Poly(ADP-ribose); Permeable cells; Inhibitors; Endonuclease; Nucleoid sedimentation.

[o]To whom correspondence should be sent

Akadémiai Kiadó, Budapest

In the process of DNA replication poly-(ADP-ribosyl)ation is regarded as a modulating reaction (Burzio and Koide, 1970; Yoshihara et al., 1975). This idea is based originally on the observation that ^{3}H-dTTP incorporation into DNA is prevented by NAD in isolated rat-liver nuclei (Burzio and Koide, 1970). On the other hand in vitro DNA synthesis in nuclei from Novikoff hepatoma (Burzio and Koide, 1972) and lymphocytes Lehmann and Shall, 1972) was not supressed on preincubation with NAD to induce ADP-ribolysation. Moreover, there are reports indicating a stimulatory influence of ADP-ribolysation on DNA synthesis upon preincubation with NAD in nuclei isolated from Hela cells (Roberts et al., 1973; Smulson et al., 1975). DNA synthesis in nuclei of chick embryo liver was stimulated by ADP-ribolysation while it was depressed in hen liver nuclei (Tanigawa et al., 1978a; Tanigawa et al., 1978b).

These controversial results prompted us to look into the possible relationship between poly(ADP-ribose) and DNA synthesis in a system that is closer to the physiological state than those of isolated nuclei. For this purpose we used reversibly permeabilized lymphocytes isolated from mouse thymus; a quasi in vivo system suitable for the study of replicative DNA synthesis (Bánfalvi et al., 1984) as well as for the synthesis of poly(ADP-ribose).

Here we discribe that the blocking either synthetic process does not inhibit the other one. Disparity in conditions for the synthesis of DNA and that of poly(ADP-ribose) indicate an indirect correlation between the processes in question.

MATERIALS AND METHODS

Chemicals

Unlabelled nucleotides included ATP, dATP, dCTP and dGTP (Calbiochem), nalidixic acid, concanavalin A and ethylenglycol-bis-(2-amino ethyleter)-N,N'-tetra aceticacid (EGTA), (SERVA), novobiocin and propidium iodine (Sigma), ^{3}H-thymidine 5'-triphosphate specific activity 26.7 Ci/mmol (Research Production and Utilization of Radioisotopes, Prague), ^{3}H-thymidine specific activity 80 Ci/mmol (New England Nuclear). Human AB serum was obtained from the Blood Transfusion Center (Budapest).

Cells

Isolation and permeabilization of mouse thymus cells were described earlier (Bánfalvi et al., 1984). The distribution of cells representing different phases of cell cycle was measured after fixing freshly isolated cells in increasing concentration of ethanol up to 70% (v/v) and by determination of DNA content based on staining with propidium iodine. For cell sorting a Beckman-Dickson FACS III system was applied according to Szabó et al. (1981). Isolated cells ($5x10^6$) were cultured in 1 ml of Eagle medium in sterile culture tubes (9x1 cm) containing 2 mM glutamin, 50 U/ml benzylpenicillin, 50 µg/ml streptomycin and 10% human AB serum inactivated at 56°C for 30 minutes. Cells were incubated at 37°C, 5% CO_2, 95% relative humidity in an Assab T-304 incubator. Nucleotid sedimentation of intact and permeabilized thymocytes was carried out on neutral sucrose gradient (Cook and Brazzel, 1975).

DNA synthesis in intact cells

Lymphocytes ($2x10^6$) were suspended in 0.5 ml Eagle's medium and incubated in the presence of 13 µM ^{3}H-thymidine (specific activity 80 Ci/mmol) at 37°C for 30 minutes. The reaction was stopped by the addition of an equal volume of 1 M perchloric acid. After centrifugation the precipitate was washed three times with cold 0.5 M perchloric acid. The precipitate was hydrolyzed in 0.5 M perchloric acid at 90°C for 30 minutes and the radioactivity of the DNA was determined by counting in a Beckman scintillation counter.

DNA synthesis in permeable cells

Permeabilized thymocytes ($2x10^6$) were sedimented at 400 g and the supernatant was removed by suction and the cells were resuspended in 50 µl permeabilization buffer containing 9 mM HEPES (pH 7.8), 5.8 mM dithiotreitol, 4.5% dextran T-150, 1 mM EGTA and 4.5 mM $MgCl_2$ and 25 µl incorporation mixture. The latter contained 100 mM HEPES (pH 7.8), 210 mM NaCl, 15 mM ATP, 0.3 mM each of dATP, dCTP, dGTP and 18 µM ^{3}H-dTTP (specific activity 0.8 Ci/mmol). ^{3}H-dTTP incorporation was allowed for 10 minutes at 37°C. The reaction was terminated and the radioactivity measured.

Poly(ADP-ribose) synthesis in permeable thymocytes

The incorporation mixture (25 µl) was given to $2x10^6$ cells suspended in 50 µl permeabilization buffer. The incorporation mixture contained: 100 mM Tris-HCl (pH 7.8), 120 mM $MgCl_2$, 150 µM ^{3}H-NAD (1 µCi per reaction). Incubation was carried out at 25°C for 10 minutes unless otherwise stated. The reaction was terminated and the radioactivity measured.

RESULTS

Distribution of isolated thymocytes

Freshly isolated mouse thymocytes comprise a heterogeneous population of T cells being in different stages of

the cell cycle. To characterize cells that are in discrete phases of orderly integrated biochemical events we determined the distribution of cell population on the basis of their DNA content using fluorometric cell sorting (Fig. 1). About 10% of the freshly isolated thymus cells are in the synthetic (S) phase of cell cycle, 10% in the DNA post synthetic phase (G_2) or in mitotic phase (M) but most of the cells (80%) were found in the G_1 or in the resting G_o phase (Fig. 1).

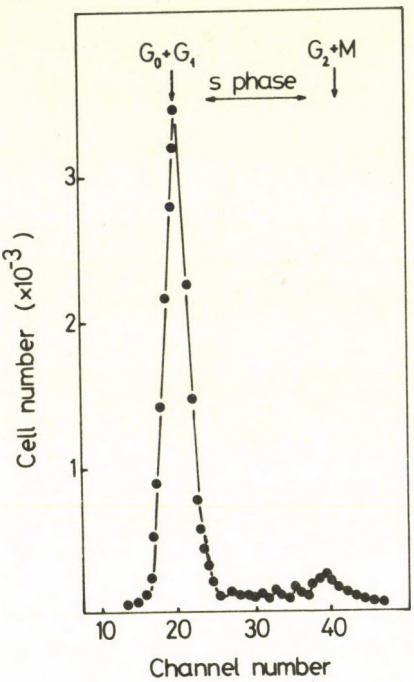


Fig. 1. Distribution of thymus cells as a function of cell cycle. Cells were fixed in alcohol and stained with propidium iodine and separated in a Beckman-Dickson FACS III cell sorter.

To test whether or not significant changes occur in the DNA structure during the procedure of permeabilization we compared the compactness of chromatin structure of intact and permeabilized thymocytes carrying out nucleoid sedimentation

analysis. We found only moderate change in the sedimentation pattern of permeabilized cells compared to the intact ones (Fig. 2).

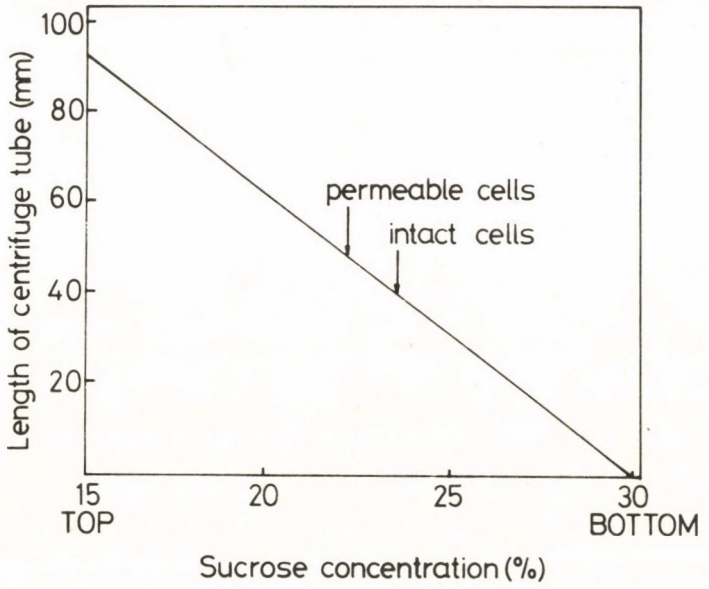


Fig. 2. Nucleotide sedimentation analysis of intact and permeabilized thymocytes. Intact and permeabilized cells ($2x10^6$ each) were layered directly on top of 15-30% linear neutral sucrose gradients. Centrifugation was for 2 h at 30 000 rpm, 20°C, in the Beckman SW 41 rotor. The position of nucleoid inside the gradient was recorded by measuring the absorbance at 260 nm.

Optimal conditions for DNA and poly(ADP-ribose) synthesis

Optimal incorporation of ^{3}H-NAD into (ADP-ribose) was reported at lower temperature than the body temperature of mammals (Giri et al., 1978; Berger et al., 1978; Altmann et al., 1981; Thi Man and Shall, 1982; Tanuma and Kanai, 1982).

We measured ^{3}H-NAD incorporation in permeable thymocytes at different temperatures for the homopolymer formation. When 150 µM NAD concentration was used, the temperature optimum was 25°C and when higher NAD concentration (1 mM) was added to the reaction mixture 37°C was the optimum (Fig. 3A). The

average NAD concentration inside lymphocytes is 0.35 mM (Berger et al., 1982).

Optimal rate for DNA synthesis was found at 37°C (Fig. 3B). Higher and lower temperatures slowed down the incorporation of ^{3}H-dTTP in accordance with typical temperature kinetics of enzymatic reactions.

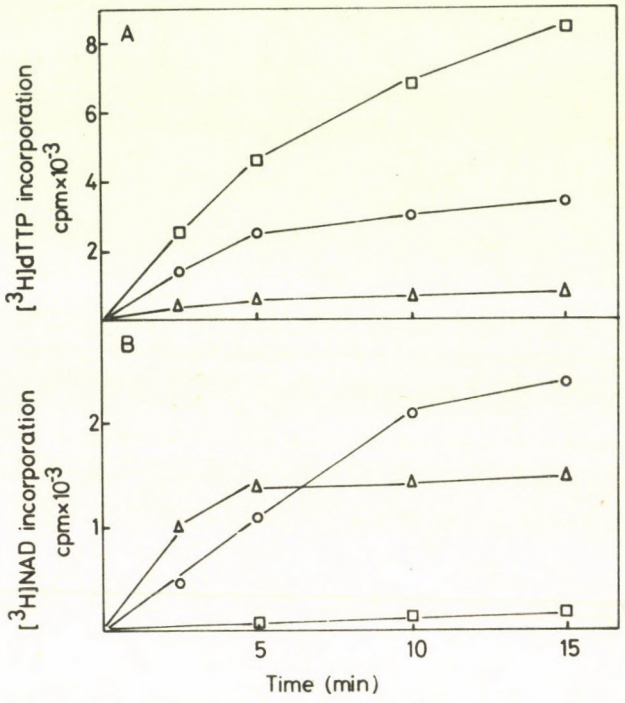


Fig. 3. Temperature optima for DNA and poly(ADP-ribose) synthesis. Thymus cells ($2x10^{6}$) were permeabilized in the presence of 4.5% dextran T-150 for 15 minutes. (A): poly(ADP-ribose) synthesis was assayed at different temperatures. Incubations were at 25°C (□), 37°C (o) and 42°C (Δ) for the time periods indicated. (B): ^{3}H-dTTP incorporation was simultaneously followed.

Bivalent cations are required in several nuclear reactions and activate Ca^{2+} and Mg^{2+} dependent endonuclease that is prone to generate cleavage sites activating poly(ADP-ribose) polymerase (Yoshihara et al., 1975) that may influence

Table 1. Effect of Mg^{2+} concentration on DNA and poly(ADP-ribose) synthesis in permeable thymocytes

Mg^{2+} mM	Incorporation ^{3}H-dTTP	^{3}H-NAD
3	100	41
5	72	-
10	33	47
20	-	57
40	12	100

Incubations were carried out in permeable cells under standard conditions of DNA synthesis described in Materials and Methods. Mg^{2+} concentration varied as indicated. Results are given as percentages of the radioactivity of maximal incorporation of ^{3}H-labeled substrates.

DNA synthesis. This possibility was tested by measuring simultaneously the rate of DNA and poly(ADP-ribose) synthesis as a function of Mg^{2+} concentration (Table 1). We detected an ion concentration dependent increase of ^{3}H-NAD incorporation with an optimum at 40 mM Mg^{2+} concentration. An increase of poly(ADP-ribose) synthesis was observed when Ca^{2+} was present in addition to Mg^{2+} ions (data not shown). A declining incorporation of ^{3}H-dTTP into DNA was registered above 3 mM Mg^{2+} concentration (Table 1).

Effect of inhibitors and substrate analogues on biopolymer synthesis

Novobiocin and nalidixic acid are specific inhibitors of bacterial gyrase and DNA topoisomerase type II enzymes of eucaryotic cells. They strongly inhibit DNA synthesis in mammalian cells (Mattern and Painter, 1979; Nishio and Uyeki, 1982). Novobiocin is supposed to bind to the ATP-binding

site of topoisomerase II preventing conformational changes necessary for DNA supercoiling and for DNA synthesis. In contrast, neither ATP-dependent topoisomerase (type II enzyme) nor ATP-independent topoisomerase (type I enzyme) were inhibited by nalidixic acid in the hands of Hsiek and Brutlag (1980).

We determined the rate of DNA synthesis in permeable thymocytes in the presence of 1 mM novobiocin. This resulted in a slight (20%) decrease in ^{3}H-NAD incorporation. Nalidix acid in 10 mM concentration also strongly inhibits DNA synthesis but not the incorporation of ^{3}H-NAD into poly(ADP-ribose) (Table 2).

We also tried to influence DNA synthesis through the inhibition of poly(ADP-ribose) polymerase. Nicotinamide, an inhibitor of poly(ADP-ribose) polymerase, (Clark et al., 1971) was used in 3 mM concentration. This concentration of nicotinamide caused an immediate block in ^{3}H-NAD incorporation. Contrary to this observation DNA synthesis represented by the incorporation of ^{3}H-dTTP was practically undisturbed whether or not nicotinamide was present in the incubation mixture (Table 2).

We have found a moderate inhibition of DNA synthesis by nicotinamide in freshly isolated and permeabilized thymocytes and in cultured intact cells. Freshly isolated thymus cells maintain a residual capacity of DNA synthesis (Bánfalvi et al., 1984) originating from in vivo stimulation. Cultured lymphocytes in resting phase do not synthesize DNA unless stimulated with mitogens such as phytohemagglutinin or concanavalin A. Nicotinamide caused an inhibition of poly(ADP-ribose) synthesis in cultured thymocytes stimulated with concanavalin A (Fig. 4). The DNA synthesis in cultured intact cells was followed by the incorporation of ^{3}H-thymidine. DNA synthesis was only slightly reduced in the presence of nicotinamide in cultured cells compared to the drastic depletion of NAD incorporation.

Table 2. Effect of inhibitors on poly(ADP-ribose) and DNA synthesis in permeable cells

Inhibitor	mM	Incorporation of ^{3}H-dTTP	Incorporation of ^{3}H-NAD
None	-	100	100
Nicotinamide	3.0	92	2
Novobiocin	0.5	27	88
	1.0	2	78
Nalidixic acid	1.0	75	110
	10.0	33	117

Macromolecular synthesis was carried out in permeable cells ($2x10^{6}$) under standard conditions described for DNA and for poly(ADP-ribose), respectively.
Inhibitors were added to the incubation mixtures as indicated. Data are averages of 3 parallel incubations. Results are given as percentages of the activity of the control samples measured in the absence of inhibitor (top line).

DISCUSSION

We studied replicative DNA synthesis along with poly(ADP-ribose) synthesis in a random population of lymphocytes isolated from mouse thymus and permeabilized in a hypotonic medium in the presence of dextran T-150. Reversibly permeabilized thymocytes provided a suitable semi in vivo system to follow DNA synthesis. In this system ^{3}H-dTTP incorporated into acid-soluble material linearly for 10 minutes and at gradually decreasing rate for an additional 20 minutes (Bánfalvi et al., 1984). Thus the relationship studied concerns only early events of synthesis of the polymers. To characterize further this system nucleoid sedimentation analysis was carried out. The analysis confirmed our earlier observation that permeabilization did not cause considerable degradation of DNA and revealed that the chromatin structure in permeable cells is close to the physiological state.

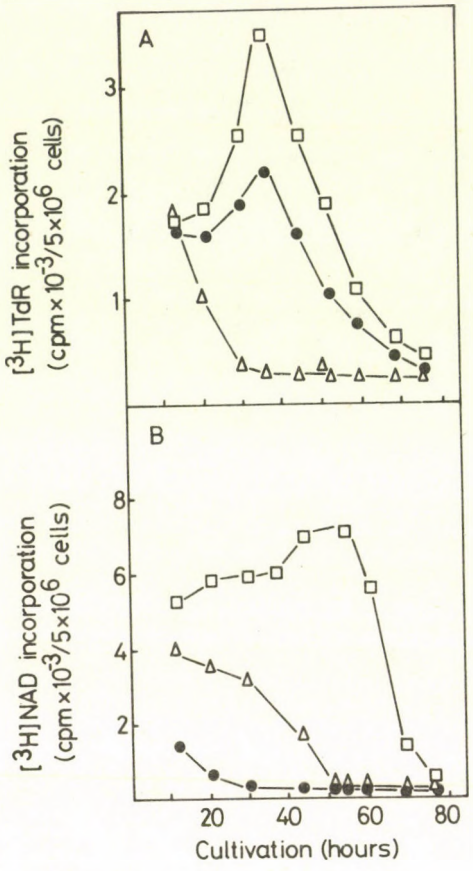


Fig. 4. Effect of nicotinamide treatment on mitogen induction of thymus cells. Cultured cells were assayed for (A) DNA and (B) for poly(ADP-ribose) synthesis during their cultivation. Poly(ADP-ribose) synthesis was carried out in permeable cells while DNA synthesis was followed by the incorporation of ^{3}H-thymidine. Incorporation of ^{3}H labeled precursor into non-stimulated cells (Δ); stimulated with 20 µg/ml concanavalin A (□); or with 20 µg/ml concanavalin A in the presence of 3 mM nicotinamide (●).

Although we found that optimal conditions for DNA synthesis and for poly(ADP-ribose) formation were different in permeable cells, we could find conditions satisfactory for both processes to go on with a reduced rate. Mg^{2+} and Ca^{2+} ions are likely candidates for regulatory functions in these processes. Our data are insufficient to claim an immediate relationship between the early events of DNA and poly(ADP-ribose) synthesis. It may well happen that poly(ADP-ribosyl)ation inhibits DNA synthesis at high Mg^{2+} concentrations as suggested (Burzio and Koide, 1970; Lindhal, 1982). Alternatively, Mg^{2+} and Ca^{2+} ions at higher concentrations may activate an endonuclease that by nicking DNA strands could enhance repair functions of poly(ADP-ribose) polymerase suppressing the replicative process. The involvement of Ca^{2+}/Mg^{2+}-dependent endonuclease in the repair of damaged DNA (Nomura et al., 1981) is an indication of this possibility.

We observed that the suspension of DNA synthesis by inhibitors of topoisomerase II was not paralelled by the decrease of poly(ADP-ribose) synthesis. This fact indicates that topological changes involved in DNA synthesis are independent of the homopolymer formation and suggest that DNA and poly(ADP-ribose) synthesis are topologically independent processes. The idea of initial DNA and poly(ADP-ribose) synthesis being independent processes was further strengthened by the fact that the inhibitor of poly(ADP-ribose) polymerase had no significant effect on DNA synthesis. Compared to the strong inhibitory effect of nicotinamide on poly(ADP-ribose) synthetase DNA synthesis was suppressed only moderately in permeabilized thymocytes and in intact cells.

In conclusion our view regarding the synthesis of DNA and poly(ADP-ribose) is that these processes are not closely related to each other. A possible coordination of the two processes through the regulation of Ca^{2+} and Mg^{2+} ions involving Ca^{2+} and Mg^{2+} dependent endonuclease are under investigation.

Acknowledgements

We thank for the assistance of Miss E. Krizsán, Mrs. Z. Nigovicz-Pál and Alexander Topaloglou. Experiments on fluorometric cell sorter under the guidance of Dr. G. Szabó Jr, are gratefully acknowledged.

REFERENCES

Altmann, H., Sherak, O. and Topaloglou, A. (1981) Versch. Dtsch. Ges. Rheumatol. 7, 561-564

Bánfalvi, G., Soóki-Tóth, A., Sarkar, N., Csuzi, S., Antoni, F., (1984) Eur. J. Biochem. 139, 553-559

Berger, N.A., Adams, J.W., Sikorski, G.W., Shirley, J. and Shearer, W.T. (1978) J. Clin. Invest. 62, 111-118

Berger, N.A., Berger, S.J., Sikorski, G.W. and Catino, D.H. (1982) Exp. Cell. Res. 137, 79-88

Burzio, L.O. and Koide, S.S. (1970) Biochem. Biophys. Res. Commun. 40, 1013-1020

Burzio, L.O. and Koide, S.S. (1972) FEBS Lett. 20, 29-32

Caplan, A.J. and Rosenberg, M.J. (1975) Proc. Natl. Acad. Sci. USA 72, 1852-1857

Chambou, P., Weill, J.D., Doly, J. Strosser, M.T. and Mandel, P. (1966) Biochem. Biophys. Res. Commun. 25, 638-643

Clark, J.B., Ferris, G.M. and Pinder, S. (1971) Biochim. Biophys. Acta 238, 82-85

Cook, P.R. and Brazzel, P.A. (1975) J. Cell. Sci. 19, 261-279

Furneaux, H.M. and Pearson, C.K. (1978) Biochem. Soc. Trans. 6, 753-755

Giri, Ch.P., West, M.H.P., Ramirez, M.L. and Smulson, M. (1978) Biochemistry 17, 3501-3504

Hayashi, O. and Ueda, K. (1977) Annu. Rev. Biochem. 46, 95-116

Hsiek, T. and Brutlag, D. (1980) Cell 21, 115-125

Kitamura, A., Tanigawa, Y., Yamamoto, T., Kawamura, D., Doi, S. and Shimoyama, M. (1979) Biochem. Biophys. Res. Commun. 87, 725-733

Lehmann, A.R. and Shall, S. (1972) FEBS Lett. 26, 181-184

Lindhal, T. (1982) Nature 298, 424-425

Mattern, M.R. and Painter, R.B. (1979) Biochim. Biophys. Acta 563, 306-312

Muller, W.E.G., Tetsuka, A., Nusser, I., Obermeier, J., Rhode, H.J. and Zahn, R.K. (1974) Nucleic Acids. Res. 1, 1317-1327

Muller, W.E.G. and Zahn, R.K. (1976) Mol. Cell. Biochem. 12, 147-159

Nishio, A. and Uyeki, E. (1982) Biochem. Biophys. Res. Commun. 106, 1448-1455

Nomura, H., Tanigawa, Y., Kitamura, A., Kawakami, K. and Shimoyama, M. (1981) Biochem. Biophys. Res. Commun. 98, 806-814

Purnell, M.R., Stone, P.R. and Wish, W.J.D. (1980) Biochem. Soc. Trans. 8, 215-227

Roberts, H.J., Stark, P. and Smulson, M.E. (1973) Biochem. Biophys. Res. Commun. 52, 43-50

Smulson, M.E., Stark, P., Gazzoli, M. and Roberts, J. (1975) Exp. Cell. Res. 90, 175-182

Szabó, G.Jr., Kiss, A. and Damjanovich, S. (1981) Cytometry 2, 20-23

Tanigawa, Y., Kawamura, M., Kitamura, A. and Shimoyama, M. (1978a) Biochem. Biophys. Res. Commun. 81, 1278-1285

Tanigawa, Y., Kitamura, A. and Shimoyama, M. (1978b) Eur. J. Biochem. 92, 261-269

Tanuma, S. and Kanai, Y. (1982) J. Biol. Chem. 257, 6565-6570

Thi Man, N. and Shall, S. (1982) Eur. J. Biochem. 126, 83-88

Yoshihara, K. and Koide, S.S. (1973) FEBS Lett. 35, 262-264

Yoshihara, K., Tanigawa, Y., Burzio, L.O. and Koide, S.S. (1975) Proc. Natl. Acad. Sci. USA 72, 289-293

Acta Biochim. Biophys. Hung. 21(4), 327-333 (1986)

A NOVEL METHOD FOR THE ISOLATION OF CARBOXYPEPTIDASE B

Erzsébet Dala, *Aranka Kiss, P. Südi and B. Szajáni

Reanal Factory of Laboratory Chemicals, Budapest, Hungary and *Department of Biochemistry, A. József University, Szeged, Hungary

(Received June 6, 1986)

SUMMARY

Carboxypeptidase B was isolated from porcine pancreas by selective heat treatment of the autolyzed tissue at 60°C and pH 6.0 the presence of phosphate ions. The heat treatment was followed by ammonium sulfate fractionation and ion-exchange in a batch system. The method could also be used for the isolation of carboxypeptidase B from beef pancreas.

INTRODUCTION

Carboxypeptidase B (EC 3.4.17.2) is used in protein biochemistry for the selective splitting of carboxy-terminal lysine and arginine from peptides and proteins. It is usually isolated from porcine pancreas (Folk et al., 1960), but methods have been published for the isolation of bovine (Wintersberger et al., 1962), goat (Dua et al., 1973; 1976; 1981; Rao and Dua, 1975) and human (Marinkovic et al., 1977) enzyme, too. All authors used the acetone powder of the pancreas or the pancreas juice as a starting material. Such steps, however, have number of shortcomings: the use of organic solvents (acetone, ether, etc.) may lead to denaturation of the enzyme.

We worked out a simple method for the isolation of carboxypeptidase B from porcine pancreas. The protective effect of the high protein and lipid content of the autolyzed organ allowed us to use selective heat treatment to separate the contami-

Abbreviation: PMSF, phenylmethylsulfonylfluoride

nating proteins without extensive denaturation of the enzyme. In addition application of ammonium sulfate fractionation and batch adsorption and elution from DEAE-cellulose yielded a product of high specific activity.

MATERIALS AND METHODS

Porcine pancreas was collected freshly in a slaughterhouse and was immediately used or stored in frozen state at -20°C until use.

Ammonium sulfate special quality and phenylmethyl-sulfonyl fluoride were purchased from Serva Feinbiochemica (Heidelberg, FRG). DEAE-cellulose and all other chemicals were of reagent grade (Reanal Factory of Laboratory Chemicals, Budapest, Hungary). Autolysis of porcine pancreas was carried out according to Folk et al. (1960) with some modifications (see in Results). Protein concentration was determined by the method of Lowry et al. (1951) as modified by Schacterle and Pollack (1973) with bovine serum albumin as standard.

Carboxypeptidase B activity was measured according to Folk et al. (1960) using hippuryl-L-arginine as substrate. One unit of enzyme activity is defined as the amount of enzyme hydrolyzing one micromole of hippuryl-L-arginine per minute at 25°C and pH 7.65.

Polyacrylamide gel electrophoresis was performed in the discontinuous buffer system described by Ornstein (1964) and Davis (1964). The total monomer concentration (acrylamide + N,N'-methylenebisacrylamide) in the running gels was 10%, the concentration of N,N'-methylenebisacrylamide being 5% of the total monomer concentration. Samples of 400 µg protein in 20% sucrose solution were applied onto the gels. Electrophoresis was carried out for 2 h at 5 mA per gel. The gels were stained in a 1% Amido black 10B solution dissolved in 7% acetic acid.

RESULTS

Autolysis of porcine pancreas

One kilogram porcine pancreas was ground and frozen. The frozen pancreas was brought to room temperature and allowed to autolyze overnight. Specific activity was determined after centrifugation from the combined supernatant and washing solutions.

Heat treatment of the autolyzate

For determination of the optimum temperature and time of the heat treatment, 0.25 kg portions of autolyzate were suspended in 250 ml of 0.05 M potassium phosphate buffer (pH 7.0)

and were kept at 50 to 75°C for 5 to 30 min. In the followings treatments will be described for 0.25 kg autolyzate suspended in 250 ml buffer. After heat treatment, 500 ml water of 0-4°C was added and the suspensions were centrifuged. The specific activity of carboxypeptidase B was determined in the supernatant. A 20 min treatment at 60°C proved to be the most effective for the extraction procedure.

Effect of ionic milieu on heat treatment of the autolyzate

The optimum ionic milieu for heat treatment was determined as follows: autolyzed pancreas suspended in buffers was kept at 60°C for 20 min. The pH varied between pH 5.0 and pH 8.5. Heat treatment and other manipulations were the same as described above. As shown in Fig. 1 the optimum pH for the heat treatment is around pH 6.0 and phosphate ions exert a strong stabilizing effect. The following purification steps were performed with the supernatants of autolyzates treated at 60°C in 0.05 M potassium phosphate at pH 6.0 for 20 min.

Optimum ammonium sulfate concentrations for fractionation

The optimum ammonium sulfate concentration for fractionation was also determined. The heat treated autolyzate was fractionated with ammonium sulfate in two consecutive steps. After the second step the specific activity of carboxypeptidase B was determined in the dialysed solution of the precipitate. Consecutive fractionation with 320 and 350 g/l ammonium sulfate proved to be the best for purification.

Isolation of porcine pancreas carboxypeptidase B

Heat treatment. 1 kg of autolyzed pancreas was suspended in 1 liter of 0.05 M potassium phosphate buffer (pH 6.0). The suspension was heated to 60°C in a boiling water bath under continuous stirring and was kept at this temperature for 20 min. Immediately after heat treatment 2 l water of 0-4°C was added and the suspension was cooled to 0°C in an ice bath. The suspension was centrifuged at 0°C at 2800 x g for 30 min. The thick layer of fat was removed from the surface and the supernatant was filtered on paper (MM 614, Macherey-Nagel, Düren, FRG) at 4°C.

Ammonium sulfate fractionation. The supernatant was cooled to 0°C and solid ammonium sulfate to 320 g/l concentration was gradually added under stirring. The suspension was kept overnight at 4°C and the precipitate was removed by centrifugation (30 min, 2800 x g).

The supernatant was brought to 350 g/l final concentration by further addition of solid ammonium sulfate under continuous stirring. The suspension was again kept overnight at 4°C and was centrifuged at 2800 x g for 30 min. The supernatant was discarded and the pellet was dissolved at 0°C in 30-40 ml of 0.05 M Tris-HCl buffer (pH 7.0) containing 6 μM PMSF to protect the enzyme against residual proteolytic activity of serine proteinases. The solution was dialyzed against 0.005 M Tris-HCl buffer, pH 7.25.

Purification by ion exchange. Ion exchange in a batch system was performed according to Folk et al. (1960) with slight modifications. To 10 g of DEAE-cellulose equilibrated with 0.005 M Tris-HCl buffer, pH 7.25, 30 ml of carboxypeptidase B solution was added and kept overnight at 4°C. The solution was removed by suction, the resin was washed with the equilibrating buffer and the active fraction was eluted with 140 ml of 0.005 M Tris-HCl buffer (pH 7.25) containing 0.3 M NaCl.

The preparation was stored at -20°C. As seen in Table 1 a 190-fold purification was achieved yielding 230 units of carboxypeptidase B per mg protein.

The specific activities of the preparations varied between 200 to 250 units/mg. The preparation contained negligible quantity of contaminating proteins only as judged by gel electrophoresis using overloaded gels (Fig. 2).

Isolation of beef pancreas carboxypeptidase B using heat treatment

Heat treatment was tested during the isolation of carboxypeptidase B from beef pancreas. From the procedure described above the ion exchange step was omitted. From 1 kg of beef pancreas 240 mg of carboxypeptidase B was obtained with a specific activity of 15.4 units/mg protein.

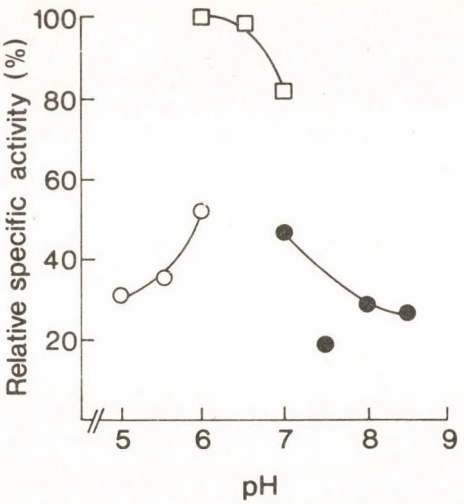


Fig. 1. (left) Effect of ionic milieu on the heat treatment of autolyzed porcine pancreas. 0.25 kg portions of autolyzed pancreas were incubated at 60°C for 20 min in the following buffers: o: 0.05 M sodium acetate; □: 0.05 M potassium phosphate; ●: 0.05 M Tris-HCl. The activity of the best preparation was taken as 100%.

Fig. 2. (right) Polyacrylamide gel electrophoretic pattern of purified porcine carboxypeptidase B. 400 µg of protein was applied onto the cylindrical gel. For experimental details see Materials and methods.

DISCUSSION

The methods described so far for the isolation of carboxypeptidase B pancreas of various mammals e.g. porcine (Folk et al., 1960), beef (Wintersberger et al., 1962), goat (Dua et al., 1973; 1981; Rao and Dua, 1975) and human (Marinkovic et al., 1977) have had the disadvantage of enzyme protein denaturation, relatively high cost, fire-risk and tediousness. To overcome these drawbacks we introduced a selective heat treatment which previously proved to be useful for the preparation of aminoacylase (Szajáni, 1980).

It was found that by heat treatment of autolyzed pancreas the preparation of acetone powder can be avoided. By a two-step ammonium sulfate fractionation followed by ion exchange

Table 1. Isolation of carboxypeptidase B from porcine pancreas

In the experiment 1 kg pancreas was processed

Purification step	Volume ml	Total enzyme activity units x 10^{-3}	Yield %	Total protein g	Specific activity units/mg protein	Purification fold
Autolyzed organ*	-	440	100	386	1.1	1.0
Heat treatment (60°C, 20 min, 2800 x g supernatant)	2900	319	72.5	131	2.4	2.2
$(NH_4)_2SO_4$ fractionation 320-350 g/l; dialyzed solution of the precipitate	45	46.4	10.5	0.29	160.0	145.5
Ion exchange in batch (DEAE-cellulose)	32	15.1	3.4	0.06	251.7	228.8

*The values presented were determined from the combined supernatant (1600 ml) and washing solutions of the autolyzed tissue (3700 ml).

of the heat-treated material in a batch system an enzyme preparation of high specific activity of 230 units/mg protein surpassed the quality of preparations described by Folk et al. (1960) who found a specific activity of 184.2 units/mg protein, or that of Ager and Hass (1977); 188 units/mg protein, and that of Sokolowsky and Zisapel (1971); about 190-195 units/mg protein for porcine pancreas. Heat treatment seems to be a useful step for isolation of carboxypeptidase B not only from porcine but from the pancreatse of other mammals, as demonstrated for the bovine enzyme.

Acknowledgements

The authors express their gratitude to Dr Gertrud Szabolcsi for her valuable suggestions and help in the preparation of the manuscript.

REFERENCES

Ager, S.P. and Hass, G.M. (1977) Anal. Biochem. 83, 285-295

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427

Dua, R.D. and Dixit, A. (1973) Ind. J. Biochem. Biophys. 10, 223-224

Dua, R.D., Anand, R.C., Altaf Hussain and Rao, M.P. (1976) Ind. J. Biochem. Biophys. 13, 106-112

Dua, R.D. and Srivastava, S.K. (1981) J. Mol. Catal. 10, 253-268

Folk, J.E., Piez, K.A., Carrol, W.R. and Gladner, J.A. (1960) J. Biol. Chem. 235, 2272-2277

Lowry, O.H., Rosebrough, N.J., Farr, L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Marinkovic, D.V., Marinkovic, J.N., Erdős, E.G. and Robinson, C.J.C. (1977) Biochem. J. 163, 253-260

Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321-349

Rao, M.P. and Dua, P.D. (1976) Ind. J. Biochem. Biophys. 12, 24-27

Schacterle, G.R. and Pollack, R.L. (1973) Anal. Biochem. 51, 654-655

Sokolowsky, M. and Zisapel, N. (1971) Biochim. Biophys. Acta 250, 203

Szajáni, B. (1980) Acta Biochim. Biophys. Acad. Sci. Hung. 15, 287-293

Wintersberger, E., Cox, D.J. and Neurath, H. (1962) Biochemistry 1, 1069-1078

Acta Biochim. Biophys. Hung. 21(4), 335-348 (1986)

ACTIVE CENTRE STUDIES ON BOVINE PANCREATIC CHYMOTRYPSIN WITH TRIPEPTIDYL-P-NITROANILIDE SUBSTRATES

József Tőzsér, Gabriella Cs.-Szabó, Marianne Pozsgay
Leif Aurell* and Pál Elődi

Department of Biochemistry, University Medical School of Debrecen, Debrecen, H-4012, Hungary
*KABI Peptide Research Laboratory, Mölndal, Sweden

(Received September 5, 1986)

SUMMARY

The kinetic behaviour of bovine pancreatic chymotrypsin was studied with 22 N-protected and 17 N-unprotected tripeptidyl-p-nitroanilide substrates. The contribution of the individual side chains to the kinetic parameters were calculated by regression analysis. At subsite P_1 (notation of Schechter and Berger, 1967, Biochem. Biophys. Res. Commun. 27, 157) Tyr seems to be better than Phe and Trp, concerning k_{cat} values. At P_2 subsite the best K_M values were obtained with Gly and Ser, whereas the hydrophobicity of P_2 subsite appears to be necessary for efficient catalytic activity. At P_3 mainly polar amino acids, both with D and L configuration, were tested. They improve the solubility of substrates in aqueous medium, as well as the kinetic parameters. Suc(OMe) and Suc protecting groups at P_4 increase significantly the catalytic activity compared to the aromatic ones. The obtained data were compared to the known substrate binding site of bovine pancreatic chymotrypsin.

Abbreviations: Ahx, 2-aminohexanoic acid; Me_2SO, dimethylsulfoxide; Phe(Gu)-, 4-guanidino-phenylalanyl-; Suc-, succinyl-; Suc(OMe)-, methoxy-succinyl-; TLCK, tosyl-L-lysine chloromethyl ketone; -NH-Np, 4-nitroanilide; PhCO-, Benzoyl-; Z-, benzyloxycarbonyl-

Correspondence to: Dr. Pál Elődi
Department of Biochemistry
University Medical School of Debrecen
Debrecen, POB 6
H-4012 Hungary

Akadémiai Kiadó, Budapest

INTRODUCTION

Although pancreatic chymotrypsin (E.C.3.4.21.4.) is the most frequently studied proteolytic enzyme, our knowledge on its specificity is far from being complete. The detailed X-ray structure of α-chymotrypsin was described by Sigler et al. (1968). Investigations with tripeptidyl-chloromethyl ketones (Segal et al., 1971), amide and ester substrates of various size (Yamamoto and Izumiya, 1966; Segal, 1972; Bauman et al., 1973; Kasafirek et al., 1976; 1980; Hill and Tomalin, 1981; Boudier et al., 1981) provided information about the enzyme-substrate interaction.

Our earlier investigations on tripeptidyl-p-nitroanilide substrates carried out with subtilisin (Pozsgay et al., 1979), porcine pancreatic elastase (Cs.-Szabó et al., 1980a), human plasmin (Cs.-Szabó et al., 1980b), human thrombin (Pozsgay et al., 1981a), pancreatic trypsin (Pozsgay et al., 1981b) suggested that the regression analysis of the kinetic parameters obtained with substrates of various amino acid sequence could be successful for predicting the "optimal" sequence of substrates. In these analyses the reciprocal values of Michaelis constant ($1/K_M$), as well as catalytic constant (k_{cat}) and proteolytic constant (k_{cat}/K_M) were subjected to regression analysis. In this way the contribution of individual substituents and protecting groups to the kinetic parameters were established. However, the drawback of these calculations is that there is apparently no physical meaning of the contributions, although the obtained values fulfill the mathematical requirements of the regression analysis, they could be used only qualitatively. In contrast to our earlier papers (Pozsgay et al., 1979; Cs.-Szabó et al., 1980a; 1980b; Pozsgay et al., 1981a; 1981b), in the present one the logarithmic values of the figures were applied in the calculations.

MATERIALS AND METHODS

Substrates were prepared by the usual stepwise synthetic methods (Houben and Weyl, 1974). They were free of contamina-

tion as tested by thin layer adsorption chromatography (Pozsgay et al., 1978) and elementary analysis. Substrate stock solutions were prepared in Me_2SO.

Assay of Enzyme Activity

TLCK treated pancreatic chymotrypsin was purchased from SIGMA (St. Louis, Mo., Type VIII. Lot No. 70F-8000). The enzyme was found to be homogenous by polyacrylamide gel electrophoresis. Chymotrypsin stock solution was freshly prepared in 1.0 mM hydrochloric acid and kept on ice. The molar activity of the enzyme was found to be 78% by spectrophotometric titration with 2-hydroxy-5-nitro-α-toluenesulphonic acid sultone (Kézdy and Kaiser, 1970). Enzyme activity was assayed at 37°C in 50 mM Tris-HCl buffer, pH 8.0, containing 3 mM $CaCl_2$, and 7% (v/v) Me_2SO. Changes in absorption at 405 nm were recorded for 3 min. The enzyme and substrate concentrations were adjusted so that within 3 min the absorbancy changed linearly as a function of time. The substrate concentration varied between 17 μM and 1.0 mM depending on the approximate K_M values. The enzyme concentration varied between 2.7 nM and 2.7 μM.

The kinetic parameters were calculated according to Wilkinson (1961) and given in the tables as the mean of 3 independent experiments, the error was within 10%. The $\ln(1/K_M)$, $\ln(k_{cat})$ and $\ln(k_{cat}/K_M)$ values were subjected to regression analysis, according to Free and Wilson (1964) as described earlier (Pozsgay et al., 1979; Cs.-Szabó et al., 1980a; 1980b; Pozsgay et al., 1981a; 1981b).

RESULTS

Kinetic parameters of tripeptidyl-p-nitroanilide substrates

The K_M, k_{cat} and k_{cat}/K_M values of 22 N-protected and 17 unprotected tripeptidyl-p-nitroanilide substrates are listed in Table 1 and 2, respectively. It is conspicuous in Table 2 that at P_3 subsite (notation of Schechter and Berger, 1967), there are mainly D-amino acids. According to our previous experiences with other serine proteinases (Cs.-Szabó et al., 1980b; Pozsgay et al., 1981a), many of them prefer D-amino acids at this subsite. The poor kinetic behaviour of PhCO-D-Arg-Pro-Tyr-NH-Np suggests that this is true for chymotrypsin only when the terminal amino group is free, in contrast to those enzymes which accept D-amino acids at P_3 position both in N-protected and N-unprotected substrates (Cs.-Szabó et al., 1980 b; Pozsgay et al., 1981a; 1981b).

Table 1. Kinetic parameters of bovine pancreatic chymotrypsin determined with N-protected tripeptidyl-p-nitroanilide substrates

(in 50 mM Tris-HCl, 3 mM $CaCl_2$ buffer, pH 8.0, containing 7% Me_2SO at 37°C)

No.	Substrate subsites P_4	P_3	P_2	P_1	Michaelis constant K_M (mM)	Catalytic constant k_{cat} (s^{-1})	Proteolytic constant k_{cat}/K_M ($M^{-1}s^{-1}$)
1. x	PhCO	Arg	Val	Phe	0.220	2.88	13 091
2. x	Suc	Arg	Val	Phe	0.139	19.04	136 978
3. x	PhCO	Orn	Val	Phe	0.532	4.72	8 872
4. x	Suc	Ala	Val	Phe	0.210	19.15	91 190
5. x	PhCO	Arg	Pro	Phe	0.130	2.00	15 384
6. x	PhCO	Ala	Pro	Phe	0.149	2.19	14 668
7. x	Suc	Ala	Pro	Phe	0.111	17.94	161 621
8. x	PhCO	Arg	Val	Tyr	0.202	5.96	29 505
9.	Suc	Arg	Val	Tyr	0.935	19.75	18 457
10. x	Suc (OMe)	Arg	Val	Tyr	0.130	23.67	182 077
11. x	PhCO	Lys	Val	Tyr	0.352	4.58	13 011
12. x	PhCO	Arg	Pro	Tyr	0.142	5.31	37 394
13.	Suc	Arg	Pro	Tyr	0.184	28.24	153 478
14. x	Suc (OMe)	Arg	Pro	Tyr	0.094	26.66	283 617
15. x	Suc	Ala	Pro	Tyr	0.202	31.61	156 487
16.	PhCo	D-Arg	Pro	Tyr	0.758	0.16	211
17.	Z	Asp	Ahx	Ahx	0.187	0.05	267
18.	Z	Arg	Ahx	Ahx	0.106	0.59	5 666
19.	Suc	Gly	Val	Ahx	0.962	0.29	303
20.	Z	Asp	Pro	Leu	0.181	0.08	442
21.	Z	Arg	Val	Leu	0.210	0.58	2 762
22.	Z	Gly	Ala	Leu	0.289	0.29	303

x/ Substrates marked with asterisk were submitted to regression analysis

Table 2. Kinetic parameters of bovine pancreatic chymotrypsin determined with tripeptidyl-p-nitroanilides
(in 50 mM Tris-HCl, 3 mM $CaCl_2$ buffer, pH 8.0, containing 7% Me_2SO at 37°C)

No.	Substrate subsites			Michaelis constant	Catalytic constant	Proteolytic constant
	P_3	P_2	P_1	K_M (mM)	$k_{cat}(s^{-1})$	$k_{cat}/K_M(M^{-1}s^{-1})$
1.	D-Arg	Gly	Phe	0.035	0.04	1 143
2.	D-Arg	Ile	Phe	0.178	3.02	16 366
3.	D-Arg	Phe	Phe	0.334	2.65	7 910
4.	D-Arg	Ser	Phe	0.024	0.38	15 833
5.	D-Arg	Glu	Phe	0.151	0.82	5 430
6.	D-Arg	Lys	Phe	0.439	2.52	5 745
7.	D-Arg	Val	Phe	0.127	5.42	42 655
8.	Arg	Val	Phe	0.893	6.83	7 557
9.	Orn	Val	Phe	1.408	2.39	1 695
10.	Phe (Gu)	Val	Phe	0.223	0.58	2 601
11.	D-Lys	Val	Phe	0.450	3.56	7 894
12.	Ala	Pro	Phe	1.020	6.98	6 843
13.	D-Arg	Val	Tyr	0.090	7.23	80 333
14.	D-Arg	Pro	Tyr	0.053	4.78	90 189
15.	Lys	Val	Tyr	0.763	4.89	6 426
16.	D-Arg	Val	Trp	0.112	3.69	32 946
17.*	Gly	Leu	Phe	1.136	6.37	2 772

*Substrate No 17 was omitted from regression analysis

The substrates were almost completely hydrolyzed, except two cases. Only 53% of Suc-Arg-Pro-Tyr-NH-Np and 16% Suc-Arg-Val-Tyr-NH-Np was split, therefore they were omitted from the calculations.

Contribution of the subsites calculated for the N-protected and N-unprotected substrates are listed in Table 3 and 4, respectively. Substrates containing Leu and Ahx at P_1 (Table 1) were not subjected to regression analysis since the equations involving them cannot be solved.

DISCUSSION

Application of the quantitative structure-activity relationship (QSAR) analysis for peptide substrates

The hydrolysis of ester and amide substrates by chymotrypsin can be described as follows (Bender and Kézdy, 1965):

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} EA + P_1 \overset{k_3}{\rightarrow} E + P_2$$

For anilides and amides acylation is the rate-limiting step, i.e. k_{cat} and $K_M = K_s$ (Bender and Kézdy, 1965).

According to the suggestion of Free and Wilson (1964) the biological activity of analogous compounds can generally be expressed as

$$B_n = \sum_g a_{ng} + \mu \qquad (n = 1, 2, 3, \ldots N)$$

where B_n represents the biological activity of the nth compound, a_{ng} is the contribution of gth subsite of the nth molecule to the affinity and μ is the overall contribution characteristic of all N members of the analogous series.

Using the logarithmic values of K_M and k_{cat} the contribution at a certain subsite is proportional to its contribution to the energy of enzyme-substrate interaction as well as to the activation energy, respectively.

By applying the regression analysis it should be presumed that intramolecular side chain interactions in the

Table 3. Contribution of the subsites of protected tripeptidyl-p-nitroanilide substrates to the kinetic parameters
(calculated from Table 1)

Subsite	Residue	Contribution to $\ln(1/K_M)$	$\ln(k_{cat})$	$\ln(k_{cat}/K_M)$
P_4	PhCO	-0.14	-0.85	-0.99
	Suc	0.05	1.14	1.19
	Suc(OMe)	0.38*	0.69	1.08
P_3	Arg	0.20	-0.01	0.19
	Orn	-0.87	0.56	-0.31
	Ala	-0.08	-0.06	-0.14
	Lys	-0.23	-0.23	-0.46
P_2	Val	-0.19	0.06	-0.13
	Pro	0.22	-0.07	0.15
P_1	Phe	0.12	-0.35	-0.24
	Tyr	-0.12	0.41	0.28
Overall contribution (μ)		1.72	2.13	10.76

*The best contribution values are underlined

chromogenic substrates are negligible. This could be tested by the method suggested by Bajusz et al. (1978), as it was also applied for thrombin and trypsin substrates (Pozsgay et al., 1981a; 1981b). It was assumed that the ratios of kinetic parameters if A_{aa}-X_{aa}-B_{aa} and A_{aa}-Y_{aa}-B_{aa} substrate pairs, i.e. those containing only one variable substituent in the sequence, are nearly identical if the properties of X_{aa} and Y_{aa} are not influenced by A_{aa} and/or B_{aa}. In this case the

Table 4. Contribution of the subsites of unprotected tripeptidyl-p-nitroanilide substrates to the kinetic parameters
(calculated from Table 2)

Subsite	Residue	Contribution to $\ln(1/K_M)$	$\ln(k_{cat})$	$\ln(k_{cat}/K_M)$
P_3	D-Arg	<u>0.68</u>	0.19	<u>0.87</u>
	Arg	-1.27	0.42	-0.86
	Orn	-1.72	-0.63	-2.35
	Phe(Gu)	0.12	-2.05	-1.93
	D-Lys	-0.58	-0.23	-0.82
	Lys	-1.45	-0.21	-1.65
	Ala	-1.92	<u>0.86</u>	-1.08
P_2	Gly	1.21	-4.13	-2.93
	Ile	-0.42	0.19	-0.27
	Phe	-1.05	0.06	-0.99
	Ser	<u>1.59</u>	-1.89	-0.30
	Gln	-0.25	-1.12	-1.37
	Lys	-1.32	0.00	-1.31
	Val	-0.08	<u>0.77</u>	0.69
	Pro	0.45	0.35	<u>0.81</u>
P_1	Phe	-0.07	-0.03	-0.10
	Tyr	<u>0.28</u>	<u>0.26</u>	<u>0.53</u>
	Trp	0.06	-0.42	-0.36
Overall contribution (μ)		1.53	0.76	9.20

interaction energy and the activation energy can be subdivided into individual energy contributions characteristic of the side chains and the backbone.

Table 5. Effect of side chain substitutions at various subsites expressed as the ratios of kinetic constants
(calculated from Table 1 and 2)

Numbers	P_4-P_1 subsites	Ratios $1/K_M$	k_{cat}	k_{cat}/K_M
1/8	PhCO-Arg-Val-Phe/Tyr	0.92	0.48	0.44
2/9	Suc-Arg-Val-Phe/Tyr	6.73	0.96	7.42
5/12	PhCO-Arg-Pro-Phe/Tyr	1.09	0.38	0.41
7/15	Suc-Ala-Pro-Phe/Tyr	1.82	0.57	1.03
*7/13	D-Arg-Val-Phe/Tyr	0.71	0.75	0.53
1/5	PhCO-Arg-Val/Pro-Phe	0.59	1.44	0.85
4/7	Suc-Ala-Val/Pro-Phe	0.53	1.07	0.56
9/13	Suc-Arg-Val/Pro-Tyr	0.20	0.70	0.12
10/14	Suc(OMe)-Arg-Val/Pro-Tyr	0.72	0.89	0.64
*13/14	D-Arg-Val/Pro-Tyr	0.59	1.51	0.89
2/4	Suc-Arg/Ala-Val-Phe	1.51	0.99	1.50
5/6	PhCO-Arg/Ala-Val-Phe	1.15	0.91	1.07
13/15	Suc-Arg/Ala-Pro-Tyr	1.10	0.89	0.98
1/2	PhCO/Suc-Arg-Val-Phe	0.63	0.15	0.10
6/7	PhCO/Suc-Ala-Pro-Phe	0.74	0.12	0.09
8/9	PhCO/Suc-Arg-Val-Tyr	4.63	0.30	1.60
12/13	PhCO/Suc-Arg-Pro-Tyr	1.30	0.19	0.24
8/10	PhCO/Suc(OMe)-Arg-Val-Tyr	0.64	0.25	0.16
12/14	PhCO/Suc(OMe)-Arg-Pro-Tyr	0.66	0.20	0.13

*Substrates marked with asterisk were taken from Table 2

Calculating with the logarithmic values of the kinetic parameters in the Free-Wilson analysis we used the same predictions. Taking the exponentional values of the difference of two contributions at the same subsite, this value should be equal to the corresponding $1/K_M$ and k_{cat} ratios of the A_{aa}-X_{aa}-B_{aa}, A_{aa}-Y_{aa}-B_{aa} substrate pair. Therefore the contribution values enables us to predict $1/K_M$, k_{cat} and k_{cat}/K_M ratios for every substrate pair which can be created from the investigated amino acid set. If the ratio concerning X_{aa}/Y_{aa} amino acid exchange could be calculated from more than one substrate pair, the contribution difference is related to the average value of these ratios. Since it is required by the conditions of regression analysis that better substrates should have a greater values, $\ln(1/K_M)$ values were used instead $\ln(K_M)$.

Intramolecular Interactions in Substrate Molecules

The ratios of the kinetic parameters referring to amino acid exchanges at one subsite are listed in Table 5. As it is shown, these ratios are similar to an amino acid exchange in most of the cases but there are also exceptions. The irregular values were obtained in the case of substrates which were not hydrolyzed completely by the enzyme (Substrates No 9 and 13 in Table 1). It was assumed that intramolecular hydrogen-bonding may exist in these substrates between the Suc protecting group and probably the hydroxyl group of P_1 Tyr, therefore these two substrates were omitted from calculations. Concerning the nearly similar values in the ratios referring to one amino acid exchange, it is also likely that in the hydrolysis of tripeptidyl-p-nitroanilides, the presence of intramolecular interactions is only exceptional.

Subsite Interactions

P_1-S_1 interaction

The substrates containing Phe and Tyr at P_1 position have similar K_M values except when Suc protecting group is at P_4 (see Table 1 and 5), therefore only a small difference was found in their contributions (see Table 3 and 4). Tyr was

slightly better for K_M in the N-unprotected series but slightly poorer in the N-protected one. The k_{cat} values show clearly a difference, since the substrates containing Tyr at P_1 have about twice as high k_{cat} values and greater contributions (Table 3 and 4). Similar results were found by Kasafirek et al.(1976; 1980). Substrates with Trp at P_1 seem to have less good K_M and k_{cat} values than the corresponding substrates with Tyr at the same site (see Table 4). The likely basis of these observations is that hydrogen bond can be formed between the hydroxyl group of Tyr and Ser-189 at the bottom of the primary substrate binding pocket (Baumann et al., 1973). Although the k_{cat} of substrates containing Leu or Ahx at P_1 are poor compared to those having aromatic residues at the same site, their relatively great affinity is unexpected (Table 1).

P_2-S_2 interaction

It is generally accepted that aliphatic amino acids with bulky side chains produce the most favourable binding at this site through the interaction with Trp-215 and Ile-99 (Yamamoto and Izumiya, 1966; Segal, 1972; Baumann et al., 1973; Kasafirek and Bartik, 1980). However, we found that the small Gly and Ser have far better binding properties at this site (see Table 2 and 4), although the k_{cat} and therefore the k_{cat}/K_M values of these substrates are very poor compared to those with bulkier side chains. Baumann et al. (1973) also found that Gly behaves unusually at this position and produce a better binding. They predicted the effect of a nonspecific interaction between P_2 Gly and the enzyme. The exchange of Gly to a hydrophobic amino acid may abolish this interaction and introduce a specific hydrophobic one instead (Baumann et al., 1973). Due to the formation of antiparallel β-sheet structure in the enzyme-substrate complex, the P_2 side chain and the Trp may get into a close vicinity. Since a steric hindrance is likely to exist between the bulky side chain of P_1 and Trp which is not likely either for Pro or the small Gly, as well as for Ser. Another possible explanation for the unexpected behaviour is that k_3 could become rate-limiting and therefore K_M is unequal K_s, so

that K_S could be far higher than K_M. Succeeding Ser and Gly (Table 4), Pro appears to be the best in binding at it is the first concerning the values of k_{cat}/K_M at this subsite (Table 3 and 4). Most of the Pro containing substrates have trans conformation with restricted rotation ability (Fischer et al., 1984) which may be convenient for the formation of antiparallel β-sheet structure in the enzyme-substrate complex. However, bulky Val have greater contribution to the k_{cat} values (see Table 3 and 4). These findings suggest that hydrophobicity at P_2 subsite seems to be a prerequisite not for better binding but for decreasing the activation energy. The polar side chains, Gln and Lys produce poor K_M, k_{cat} and k_{cat}/K_M values (Table 4). Since the contribution of Lys to k_{cat} is similar to that of Ile and Phe, formation of hydrophobic interaction can be presumed between the $-(CH_2)_4$-moiety of Lys of the substrate and Trp-215 in the enzyme.

P_3-S_3 interaction

At P_3, except Ala and Gly, mainly polar amino acids both of D and L configuration were tested, since they increase the solubility of substrates in aqueous medium. Due to the aromatic primary specificity and the demand for hydrophobic residue at the second site for the enzyme, P_3 and P_4 seems to be suitable for this purpose. In the N-unprotected substrates the D-amino acids bring about better K_M values than the corresponding L ones, but their contributions to the k_{cat} are alike (Table 4). On the other hand, PhCO-D-Arg-Pro-Tyr-NH-Np which also contains D-amino acid at the P_3 position, provide poor kinetic parameters (Table 1) probably because the PhCO-group hinders the favourable binding of the D-amino acid. The other two basic amino acids Orn and Lys are less effective at this position (Table 3 and 4), though Orn have better contribution to k_{cat} than Arg in the N-protected series (Table 3). D-Arg in the N-unprotected and Arg in the N-protected series are favourable not only for increasing the solubility, but they also produce better K_M and k_{cat}/K_M values than the nonpolar Ala, though it is uncertain whether the guanidino group has role at all in

binding since the $-(CH_2)_3$-moiety of Arg and Orn, $-(CH_2)_4$-moiety of Lys could be involved by hydrophobic interactions either. Comparing the kinetic parameters of Z-Asp-Ahx-Ahx-Nh-Np and Z-Arg-Ahx-Ahx-NH-Np (Table 1), the negatively charged Asp diminishes about tenfold the catalytic activity compared to Arg, though it does not greatly affect K_M.

P_4-S_4 interaction

At P_4 position four protecting groups were applied. Only three of them can be compared, however, because substrates containing Z at P_4have quite different amino acid sequence comparing to the others. Suc(OMe) protecting group appeared to be the most favourable since it increased the k_{cat} similarly to Suc compared to the aromatic PhCO. It lacks negative charge, which may be partly responsible for the poor binding of substrates containing Tyr at P_1 (Table 1, substrates No 9 and 13). Kasafirek and Bartik (1980) also found Suc to be a good protecting group, though they have assumed that P_4-S_4 is if hydrophobic nature. The great k_{cat} increasing effect of Suc and Suc(OMe) does not support this idea. However, formation of hydrogen bonding between Ser-217 and $-COO^-$ of Suc or $-COOCH_3$ of Suc(OMe) seem to be a reasonable explanation for this effect.

The substrate series were designed so that the kinetic parameters could be evaluated both with simple comparison and with regression analysis. The results obtained are very much alike.

Acknowledgements

Thanks are due to Mrs Éva Nagy Feitelson for the preparation of the manuscript. This work was supported by a grant from the Hungarian Ministry of Health.

REFERENCES

Bajusz, S., Barabás, É., Tolnay, P., Széll, E. and Bagdy, D. (1978) Int. J. Peptide Protein Res. 12, 217-221

Baumann, W.K., Bizzozero, S.A. and Dutler, H. (1973) Eur. J. Biochem. 39, 381-391

Bender, M.L. and Kézdy, F.J. (1965) Ann. Rev. Biochem. 34, 49-76

Boudier, C., Jung, M.L., Stambolieva, N. and Bieth, J.G. (1981) Arch. Biochem. Biophys. 210, 790-793

Cs.-Szabó, G., Pozsgay, M., Gáspár, R. and Elődi, P. (1980a) Acta Biochim. Biophys. Acad. Sci. Hung. 15, 263-273

Cs.-Szabó, G., Pozsgay, M. and Elődi, P. (1980b) Thromb. Res. 20, 199-206

Fischer, G., Bang, H., Berger, E. and Schellenberger, A. (1984) Biochim. Biophys. Acta 791, 87-97

Free, S.M. and Wilson, J.W. (1964) J. Med. Chem. 7, 395-399

Hill, R.C. and Tomalin, G. (1981) Biochim. Biophys. Acta 660, 65-72

Houben, J. and Weyl, T.H. (1974) Methoden der Organischen Chemie, Vol. 15, Teil 1, 2. Synthese von Peptiden (Wünsch, E. ed.) Georg Thieme Vlg., Stuttgart

Kasafirek, E., Fric, P., Slaby, J. and Malis, F. (1976) Eur. J. Biochem. 69, 1-13

Kasafirek, E. and Bartik, M. (1980) Collect. Czech. Chem. Commun. 45, 442-451

Kézdy, F.J. and Kaiser, E.T. (1970) Methods Enzymol. 19, 3-20

Pozsgay, M., Gáspár, R., Bajusz, S. and Elődi, P. (1979) Eur. J. Biochem. 95, 115-119

Pozsgay, M., Cs.-Szabó, G., Bajusz, S., Simonsson, R., Gáspár, R. and Elődi, P. (1981a) Eur. J. Biochem. 115, 491-495

Pozsgay, M., Cs.-Szabó, G., Bajusz, S., Simonsson, R., Gáspár, R. and Elődi, P. (1981b) Eur. J. Biochem. 115, 497-502

Pozsgay, M., Gáspár, R. and Elődi, P. (1978) Acta Biochim. Biophys. Acad. Sci. Hung. 13, 185-188

Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162

Segal, D.M., Powers, J.C., Cohen, G.H., Davies, D.R. and Wilcox, P.E. (1971) Biochemistry 10, 3728-3738

Segal, D.M. (1972) Biochemistry 11, 349-356

Sigler, P.B., Blow, D.M., Matthews, B.W. and Henderson, R. (1968) J. Mol. Biol. 35, 143-164

Wilkinson, G.N. (1961) Biochem. J. 80, 324-332

Yamamoto, T. and Izumiya, N. (1966) Arch. Biochem. Biophys. 114, 459-464

Acta Biochim. Biophys. Hung. 21(4), 349-362 (1986)

MAPPING OF THE SUBSTRATE BINDING SITE OF HUMAN LEUKOCYTE CHYMOTRYPSIN (CATHEPSIN G) USING TRIPEPTIDYL-P-NITROANILIDE SUBSTRATES

Gabriella Cs.-Szabó, József Tőzsér, Leif Aurell[*] and Pál Elődi

Department of Biochemistry, University Medical School of Debrecen, Debrecen, H-4012, Hungary
[*]KABI Peptide Research Laboratory, Mölndal, Sweden

(Received September 5, 1986)

SUMMARY

The kinetic constants, K_M, k_{cat} and k_{cat}/K_M of human leukocyte chymotrypsin (Cathepsin G, E.C.3.4.21.20.) were determined with 33 N-protected and 16 N-unprotected tripeptidyl-p-nitroanilide substrates. The individual contributions of the amino acid side chains of the substrates at P_1-P_4 subsites to the kinetic parameters were calculated by regression analysis. As far as K_M is concerned, the highest contributions yielded the structure of an "optimum" substrate PhCO-Ala-Val-Tyr-pNA. The contribution values permitted us to characterize the S_1-S_4 binding segment in the enzyme's binding site, interacting with the P_1-P_4 moieties of the substrate. The enzyme prefers uniformly hydrophobic substituents at the S_1-S_4 sites. The S_1 primary specificity subsite of the enzyme can bind Leu and 2-aminohexanoic acid as well, besides the aromatic amino acids Phe, Tyr, Trp. Data were compared with those obtained when studying pancreatic chymotrypsin with the same substrates.

Abbreviations: HL, human leukocyte; Me_2SO, dimethyl sulfoxide; NH-Np, p-nitroaniline; -NH-Np, p-nitroanilide; Z-, benzyl-oxy-carbonyl-; PhCO, benzoyl-; Suc-, succinyl-; Suc(OMe), methoxysuccinyl-; Asp(OBut), aspartate-γ-O-buthyl ester; Phe(Gu), guanidino-phenylalanine; Ahx, 2-aminohexanoic acid; pGlu, pyroglutamic acid

Correspondence to: Dr. Pál Elődi
Department of Biochemistry
University Medical School of Debrecen
Debrecen, POB 6
H-4012 Hungary

Akadémiai Kiadó, Budapest

INTRODUCTION

The azurofil granulum fraction of human polymorphonuclear leukocytes contains, besides acidic hydrolases, neutral proteases, elastase (Janoff and Scherer, 1968) and chymotrypsin (cathepsin G) as well Schmidt and Havemann, 1974). These enzymes are usually involved in the phagocytosis and probably in the turnover of interstitial proteins. The HL elastase plays a role in several diseases, i.e. rheumatoid arthritis, inflammations, lung emphysema, etc., and destroys interstitial proteins. The role of HL chymotrypsin is unclear yet.

The HL chymotrypsin can degrade collagen (Starkey et al., 1977), cartilage proteoglycan (Feinstein et al., 1976) and lung elastin (Reilly and Travis, 1980). In the latter case it increases the elastolytic activity of HL elastase (Boudier et al., 1981).

Examining the primary specificity of the enzyme it was observed that it splits peptide bonds mainly at the carboxyl function of Phe and Tyr (Blow and Barrett, 1977; Levy and Feinstein, 1979).

To examine HL chymotrypsin peptide-p-nitroanilides (Zimmerman and Ashe, 1977; Nakajima et al., 1979; Yoshida et al., 1980; Tanaka et al., 1985), peptide amides (McRae et al., 1980), peptide thiobenzylesters (Harper et al., 1981; Harper et al., 1984) as substrates and several inhibitors like peptide-chloromethyl ketones (Powers et al., 1977; Powers et al., 1985) were studied, but the detailed structure of the substrate binding site of cathepsin G has not yet been elucidated.

In the present study the substrate specificity of HL chymotrypsin is examined using tripeptidyl-p-nitroanilide substrates having a great variety of amino acids at the subsites. To characterize the properties of the binding site kinetic data were analyzed by the mathematical method of Free and Wilson (1964) applied succesfully earlier for other serine proteinases (Pozsgay et al., 1979; Cs.-Szabó et al., 1980a; Marossy et al., 1980; Cs.-Szabó et al., 1980b; Pozsgay et al., 1981; 1981). The results are compared with those obtained by the same substrate series using pancreatic chymotrypsin (Tőzsér et al., 1986).

MATERIALS AND METHODS

HL chymotrypsin was isolated and purified from the granules of human granulocytes according to Feinstein and Janoff (1975) and the individual isozymes were not isolated and investigated separately. The investigated preparations contained the three isozymes only, as found by gelelectrophoresis. The molecular weights of isoenzymes were 21 000, 24 000 and 27 000 and it was taken as 24 000 on the average for the calculations.

Tripeptidyl-p-nitroanilide substrates

The substrates were synthesized by the conventional peptide-chemistry methods (Houben and Weyl, 1974). NH-Np was bound to the amino acids protected by Z-group by phosphazo method (Goldschmidt and Rosculet, 1960). Z-protecting group was removed with hydrogen bromide dissolved in glacial acetic acid, and the other residues were attached stepwise to the amino acid-NH-Np. The substrates appeared to be homogeneous by Kieselgel G thin-layer chromatography and elementary analysis.

Assay of enzyme activity was carried out at 37°C in 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium chloride and 7% v/v dimethyl sulfoxyde. The concentration of the enzyme varied in the nM to μM range depending on the catalytic productivity of the substrate. The substrate concentration varied between 6 μM and 5 mM. The assay of enzyme activity, the calculations and the regression analysis of kinetic constants are described in the preceeding paper (Tőzsér et al., 1986). The kinetic parameters given in the tables represent the mean of 3 independent experiments, the error was within 10%.

RESULTS

Kinetic constants determined with tripeptidyl-NH-Np substrates

Kinetic constants of HL chymotrypsin measured with the substrates and calculated according to Wilkinson (1961) are shown in Table 1 and 2. In Table 1 the kinetic constants of both N-protected substrates containing Phe, Tyr, Trp, Ahx or Leu at P_1 position (notation of Schechter and Berger, 1967), are shown. Table 1 also summarizes the substrates which were not hydrolyzed even at an enzyme concentration as high as 10 μM (substrates 22-31).

As seen in Table 1, the enzyme readily hydrolyzes the -Leu and -Ahx-NH-Np substrates (No. 16-21), but the rate of hydrolysis, i.e. k_{cat}, is much lower, than those containing aromatic residues at P_1. The enzyme is unable to hydrolyze the

Table 1. Kinetic parameters of granulocyte chymotrypsin determined with N-protected tripeptidyl-p-nitroanilide substrates

(in 50 mM Tris-HCl, 50 mM NaCl buffer, pH 8.0, containing 7% Me_2SO at 37°C)

No.		Substrate subsites				Michaelis constant	Catalytic constant	Proteolytic constant
		P_4	P_3	P_2	P_1	K_M (mM)	$k_{cat}(s^{-1})$	$k_{cat}/K_M(M^{-1}s^{-1})$
1.	x	PhCO	Arg	Val	Phe	0.079	1.96	24 810
2.	x	Suc	Arg	Val	Phe	0.383	2.47	6 450
3.	x	PhCO	Orn	Val	Phe	0.079	0.67	8 480
4.	x	Suc	Ala	Val	Phe	0.071	0.89	12 540
5.	x	PhCO	Arg	Pro	Phe	0.407	3.72	9 160
6.	x	PhCO	Ala	Pro	Phe	0.281	2.36	8 400
7.	x	Suc	Ala	Pro	Phe	0.322	3.57	10 750
8.	x	PhCO	Arg	Val	Tyr	0.041	1.35	33 050
9.		Suc	Arg	Val	Tyr	0.370	2.31	6 240
10.	x	Suc (OMe)	Arg	Val	Tyr	0.122	2.22	18 200
11.	x	PhCO	Lys	Val	Tyr	0.256	1.59	6 200
12.	x	PhCO	Arg	Pro	Tyr	0.213	2.36	11 100
13.		Suc	Arg	Pro	Tyr	1.176	5.77	4 900
14.	x	Suc (OMe)	Arg	Pro	Tyr	0.595	2.39	4 020
15.	x	Suc	Ala	Pro	Tyr	0.315	2.47	7 840
16.		Z	Asp	Ahx	Ahx	2.000	0.36	180
17.		Z	Arg	Ahx	Ahx	1.235	0.15	120
18.		Suc	Gly	Ala	Leu	1.220	0.03	25
19.		PhCO	Arg	Ahx	Ahx	0.666	0.07	105
20.		Z	Arg	Val	Leu	0.500	0.01	20
21.		PhCO	Arg	Val	Leu	0.497	0.03	60
22.		Z	Asp	Pro	Leu	0.330	0.06	180
23.		Z	Gly	Ala	Leu	0.081	0.06	740
24.		PhCO	DArg	Pro	Tyr		not hydrolyzed	
25.		Z	Leu	DPhe	Phe		"	
26.		Z	Arg	Gly	Phe		"	
27.		Z	pGlu	Gly	Phe		"	
28.		Z	Gly	Gly	Phe		"	
29.		Suc	Gly	Gly	Phe		"	
30.		Suc	Gly	Gly	Tyr		"	
31.		Z	Arg	Ahx	Phe		"	
32.		PhCO	Phe	Ahx	Phe		"	
33.		Z	Asp (OBut)	Ahx	Phe		"	

[x] Substrates marked with asterisk were submitted to regression analysis

Table 2. Kinetic constants of granulocyte chymotrypsin determined with N-unprotected tripeptidyl-p-nitroanilide substrates

(in 50 mM Tris-HCl, 50 mM NaCl buffer, pH 8.0, containing 7% Me_2SO at 37°C)

No.	Substrate subsites			Michaelis constant	Catalytic constant	Proteolytic constant
	P_3	P_2	P_1	K_M (mM)	$k_{cat}(s^{-1})$	$k_{cat}/K_M(M^{-1}s^{-1})$
1.	D-Arg	Gly	Phe	0.787	0.92	1 170
2.	D-Arg	Ile	Phe	0.321	1.97	6 140
3.	D-Arg	Phe	Phe	0.158	0.92	5 820
4.	D-Arg	Ser	Phe	0.442	3.37	7 610
5.	D-Arg	Glu	Phe	0.980	4.03	4 110
6.	D-Arg	Lys	Phe	0.022	0.93	42 270
7.	D-Arg	Val	Phe	0.188	0.63	3 350
8.	Arg	Val	Phe	0.050	0.28	5 600
9.	Orn	Val	Phe	0.307	0.39	1 270
10.	Phe(Gu)	Val	Phe	0.382	0.34	890
11.	D-Lys	Val	Phe	0.352	8.90	24 930
12.	Ala	Pro	Phe	0.292	0.26	890
13.	D-Arg	Val	Tyr	0.107	0.11	1 030
14.	D-Arg	Pro	Tyr	1.667	2.44	1 460
15.	Lys	Val	Tyr	0.452	0.79	1 750
16.	D-Arg	Val	Trp	1.010	1.33	1 320

amide bonds at the C-terminus of Gly, Ala, Val and Arg under the applied conditions, i.e. it is inactive both with elastase and trypsin type substrates. In Table 2 (in N-unprotected substrates) at P_3 subsite usually D-amino acids are found, since our earlier studies (Cs.-Szabó et al., 1980a; 1980b; Pozsgay et al., 1981a; 1981b) suggested that amino acids of D-configuration appear to be favourable for serine-proteinases

at this site. The K_M Michaelis constant of substrates are in the 100 μM order of magnitude. The values of catalytic constants of HL chymotrypsin are quite low compared to its pancreatic analogue (Tőzsér et al., 1986).

Substrate side chain interactions

When applying regression analysis, it is required that the parameters should be additive, i.e. the effect of the amino acid side chains at the substrate subsites should function independently. To control this substrate pairs differing only in a single amino acid residue are compared (Table 3) as described in the preceeding paper (Tőzsér et al., 1986).

Regression analysis of kinetic data

The analysis renders possible to determine the contribution of individual amino acid residues at the substrate subsites to the kinetic parameters. Since these contributions are independent of each other, from the measured kinetic values of 13 protected and 16 unprotected substrates (Table 1 and 2), the kinetic properties of 48 and 168 compounds can be predicted simply by the addition of individual and overall contribution values of the residues at the given subsites (Table 4 and 5).

DISCUSSION

Investigating the kinetic constants (Table 1 and 2) HL chymotrypsin seems to be a "poor catalyst". In the case of good substrates, the K_M values are in the 10 μmolar range, which corresponds to the usual Michaelis constants of other serine proteinases measured with -NH-Np substrates. The k_{cat} values, however, are low even with -Phe- and -Tyr-NH-Np substrates.

Substrate binding site of HL chymotrypsin and its comparison with the pancreatic enzyme

The data described here are compared to those as are presented in the preceeding paper (Tőzsér et al., 1986) to characterize the common and individual features of the two enzymes.

Table 3. Effect of side chain substitutions at various subsites expressed as the ratios of kinetic constants (calculated from Table 1 and 2)

Numbers	P_4-P_1 subsites	Ratios		
		$1/K_M$	k_{cat}	k_{cat}/K_M
1/8	Bz-Arg-Val-Phe/Tyr	0.52	1.45	0.75
2/9	Suc-Arg-Val-Phe/Tyr	0.97	1.06	1.03
5/12	Bz-Arg-Pro-Phe/Tyr	0.52	1.59	2.98
7/15	Suc-Ala-Pro-Phe/Tyr	0.95	1.45	1.37
*7/13	D-Arg-Val-Phe/Tyr	0.57	5.72	3.26
1/5	Bz-Arg-Val/Pro-Phe	5.15	0.53	2.71
4/7	Suc-Ala-Val/Pro-Phe	4.68	0.25	1.17
9/13	Suc-Arg-Val/Pro-Tyr	2.44	0.40	1.27
10/14	Suc(OMe)-Arg-Val/Pro-Tyr	4.88	0.57	4.53
*13/14	D-Arg-Val/Pro-Tyr	15.58	0.04	0.71
2/4	Suc-Arg/Ala-Val-Phe	0.18	2.78	0.51
5/6	Bz-Arg/Ala-Val-Phe	0.69	1.59	1.09
13/15	Suc-Arg/Ala-Pro-Tyr	0.27	2.33	0.63
1/2	Bz/Suc-Arg-Val-Phe	4.85	0.79	3.85
6/7	Bz/Suc-Ala-Pro-Phe	1.18	0.66	0.78
8/9	Bz/Suc-Arg-Val-Tyr	9.07	0.58	5.29
12/13	Bz/Suc-Arg-Pro-Tyr	5.52	0.41	2.26
8/10	Bz/Suc(OMe)-Arg-Val-Tyr	2.99	0.61	1.82
12/14	Bz/Suc(OMe)-ARg-Pro-Tyr	2.79	0.99	2.76

*Substrates marked with asterisk were taken from Table 2

Table 4. Contribution of the subsites of protected tripeptidyl-p-nitroanilide substrates to the kinetic parameters
(calculated from Table 1)

Subsite	Residue	Contribution to $\ln(1/K_M)$	$\ln(k_{cat})$	$\ln(k_{cat}/K_M)$
P_4	PhCo	0.46	-0.10	0.35
	Suc	-0.56	0.14	-0.41
	Suc(OMe)	-0.50	0.08	-0.41
P_3	Arg	-0.16	0.22	0.05
	Orn	-0.05	-0.78	-0.83
	Ala	0.78	-0.28	0.50
	Lys	-1.90	0.37	-1.53
P_2	Val	0.76	-0.32	0.44
	Pro	-0.89	0.37	-0.52
P_1	Phe	-0.31	0.13	-0.17
	Tyr	0.36	-0.15	0.20
Overall contribution (μ)		1.68	0.67	9.26

S_4-P_4 interaction

Comparison of data in Table 1 and 2 suggests that the kinetic parameters are influenced by the S_4-P_4 interaction. As far as the protective groups are concerned, the contribution of aromatic PhCO to the K_M Michaelis constant is small, whereas to k_{cat} is higher, therefore the productivity of the enzyme is greater (compare substrates 3, 6, 11 in Table 1 with 9, 12, 15 in Table 2). Group Z is also suitable for binding, but in a lesser extent than PhCO (Table 1, 17/19, 20/21 substrates). In agreement with the results of Tanaka et al.

Table 5. Contribution of the subsites of unprotected tripeptidyl-p-nitroanilide substrates to the kinetic parameters
(calculated from Table 2)

Subsite	Residue	Contribution to $\ln(1/K_M)$	$\ln(k_{cat})$	$\ln(k_{cat}/K_M)$
P_3	D-Arg	-0.02	0.08	0.06
	Arg	1.31	-0.73	0.57
	Orn	-0.51	-0.40	-0.92
	Phe(Gu)	-0.73	-0.54	-1.27
	D-Lys	-0.66	2.73	2.06
	Lys	-1.47	2.05	0.59
	Ala	2.29	-3.91	-1.61
P_2	Gly	-0.95	-0.37	-1.33
	Ile	-0.05	0.39	0.33
	Phe	0.66	-0.37	0.28
	Ser	-0.37	0.92	0.55
	Gln	-1.17	1.10	-0.07
	Lys	2.63	-0.36	2.26
	Val	0.48	-0.75	-0.27
	Pro	-2.27	2.35	0.07
P_1	Phe	0.00	0.28	0.28
	Tyr	0.57	-1.47	-0.90
	Trp	-1.68	1.03	-0.66
Overall contribution (μ)		1.22	-0.07	8.05

(1985) the polar Suc- and Suc(OMe)- groups are favourable for the catalytic constants, but they increase the K_M values (see Table 1, substrates 1, 7 and 8, 12 in Table 2). Therefore we may assume that the more polar the protective group is, the stronger is this effect (Table 4). These findings suggest that the S_4-P_4 binding-site is nonpolar, possibly favours aromatic substituents. Protective groups in the substrates may bring about favourable kinetic properties for the pancreatic enzyme, as well. This binding subsite in the two enzymes, however, appears to be somewhat different, since for the pancreatic enzyme Suc(OMe)- protecting group seemed to be superior both for k_{cat} and K_M.

S_3-P_3 interaction

At P_3 subsite the nonpolar amino acid e.g. Ala is preferred over polar ones for binding (Table 4 and 5). The favourable porperties of Ala are supported by the fact that it occurs at P_3 position in most of the effective substrates and inhibitors applied so far (Boudier et al., 1981; Zimmerman and Ashe, 1977; McRae et al., 1980). Powers et al. (1977; 1985) reported that other nonpolar amino acids Gly and Val are also convenient at P_3 subsite. As far as charged amino acids are concerned (Table 1) Arg binds better than Asp (substrates 16 and 17). According to Tanaka et al. (1985) the strength of binding follows the order of Glu>Ala>Lys, just like the catalytic constant does. The inverse relationship between K_M and k_{cat} we had already reported earlier (Cs.-Szabó et al., 1980a; 1980b). It is valid for all the subsites, that the side chain which promotes the binding, i.e. diminishes K_M, may reduce the rate of hydrolysis.

It seems reasonable to assume that the P_3 binding segment in the leukocyte enzyme is non-polar, and is probably unable to receive bulky side chains, since the Phe(Gu) side chain (Table 2, substrate 10) was unfavourable. When the P_3 subsite is occupied by charged Arg, Lys or Orn, their hydrocarbon moiety is probably involved in the binding to the enzyme. Substrates for the pancreatic enzyme containing Arg at P_3 have

nearly the same k_{cat} but lower K_M as those having Ala at this subsite. The S_3 subsite in the enzyme seems to be polar and able to receive bulky side-chains. This suggest that the two S_3 subsites are probably different.

S_2-P_2 interaction

Regarding K_M, the bulky non-polar amino acids like Phe, Val, Ile seem to be favourable (Table 4 and 5), whereas the small Gly and Pro are disadvantageous. Charged Lys side chain promotes unexpectedly well the productive binding of substrates to the enzyme. It is still to be clarified whether the long hydrocarbon chain or the $-NH_3^+$ group of Lys is responsible for this effect. In contrast, for other serine proteinases Pro is definitely preferable at P_2 position. As far as catalytic efficiency is concerned, the situation is reversed again, Pro, Ser, Gln are favoured. Pro is usually convenient at the P_2 position also in the peptide inhibitors (Zimmerman and Ashe, 1977; McRae et al., 1980).

At P_2 position amino acids of D-configuration are inconvenient (Table 1, substrate 25). The binding is also weakened by Gly and Ahx, no matter which amino acid residue follows them at P_3 subsite (substrates 26-30 and 31-33). Neither had Tanaka et al. (1985) observed hydrolysis with substrates containing Gly at P_2 position. Therefore we assume that the S_2 binding-site of the enzyme prefers to receive bulky, non-polar side chains. Concerning K_M hydrophobic side chains fit well to S_2 subsite in the pancreatic enzyme, too. The HL enzyme favours small, polar side chains, as Ser of Gln, but not Gly, whereas the pancreatic enzyme splits at a low rate substrates containing these residues, although their low K_M suggests that these might be bound strongly to the pancreatic enzyme.

Primary specificity site

Similarly to other findings (Blow and Barrett, 1977; Zimmerman and Ashe, 1977; Tanaka et al., 1985; McRae et al., 1980) we have found that cathepsin G, HL chymotrypsin is a "real" chymotrypsin, i.e. it is most effective in hydrolyzing

at the carboxyl function of aromatic side chains. As shown in Table 1, the enzyme is able to split peptide bonds formed by Leu and Ahx at a reduced rate. However, amide bonds formed by Ala, Gly, Val and Arg remain unchanged.

The S_1 segment of the enzyme is probably spacious and non-polar. Tyr seems to be the strongest in binding. Considering the rate of hydrolysis, however, Trp appears to be the best (Table 4 and 5). Concerning K_M values the two enzymes appear to be alike, except the case when Trp occupies P_1 subsite. This hinders the productive binding of the substrates to HL chymotrypsin but not to the pancreatic enzyme. When k_{cat} values are considered, the pancreatic enzyme splits at a higher rate those substrates which contain Tyr at P_1 than those having Phe at this subsite.

In conclusion we may assume that the S_1-S_4 substrate binding sites of HL chymotrypsin are mainly non-polar, and with the exception of S_3, it is ready to interact with bulky aromatic or aliphatic side chains.

Some non-polar sequences in natural compounds, e.g. -Ile-His-Pro-Phe- in angiotensinogen, are also readily split by HL chymotrypsin. In its most efficient natural inhibitor, α-anti-chymotrypsin, the -Ile-Tyr-Leu-Leu- segment is bound directly when it interacts with the enzyme. The -NH-Np derivatives of the above sequences are good substrates of the enzyme (Tanaka et al., 1985).

The primary specificity of the HL and pancreatic chymotrypsin is similar, however, their other substrate binding subsites differ slightly from each other either in polarity or space-filling properties.

Acknowledgements

The excellent technical assistance of Mrs Erzsébet Klemm, Mrs Erzsébet Zámbori and Miss Klára Faragó is gratefully acknowledged. Thanks are due to Mrs Éva Nagy Feitelson for preparation of the manuscript. This work was supported by a grant of the Hungarian Ministry of Health.

REFERENCES

Blow, A.M.J. and Barrett, A.J. (1977) Biochem. J. 161, 17-19

Boudier, C., Holle, C. and Bieth, J.G. (1981) J. Biol. Chem. 256, 10256-10258

Cs.-Szabó, G., Pozsgay, M., Gáspár, R. and Elődi, P. (1980) Acta Biochim. Biophys. Acad. Sci. Hung. 15, 263-273

Cs.-Szabó, G., Pozsgay, M. and Elődi, P. (1980) Thromb. Res. 20, 199-206

Feinstein, G., Malemud, C.J. and Janoff, A. (1976) Biochim. Biophys. Acta 429, 925-932

Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 403, 477-492

Free, S.M., Jr. and Wilson, J.W. (1964) J. Med. Chem. 7, 395-399

Goldschmidt, S. and Rosculet, G. (1960) Chem. Ber. 93, 2387-2394

Harper, J.W., Ramirez, G. and Powers, J.C. (1981) Anal. Biochem. 118, 382-387

Harper, J.W., Cook, R.R., Roberts, C.J., McLaughlin, B.H. and Powers, J.C. (1984) Biochemistry 23, 2995-3002

Houben, J. and Weyl, T.H. (1974) Methoden der organischen Chemie, Band 15, Teil 1-2, Synthese von Peptiden (Wünsch, E. ed.) Georg Thieme Vlg., Stuttgart

Janoff, A. and Scherer, J. (1968) J. Exp. Med. 128, 1137-1151

Levy, H. and Feinstein, G. (1979) Biochim. Biophys. Acta 567, 35-42

Marossy, K., Cs.-Szabó, G., Pozsgay, M. and Elődi, P. (1980) Biochem. Biophys. Res. Commun. 96, 762-769

McRae, B., Nakajima, K., Travis, J. and Powers, J.C. (1980) Biochemistry 19, 3973-3978

Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. (1979) J. Biol. Chem. 254, 4027-4032

Powers, J.C., Gupton, B.F., Harley, A.D., Nishino, N. and Whitley, R.J. (1977) Biochim. Biophys. Acta 485, 156-166

Powers, J.C., Tanaka, T., Harper, J.W., Minematsu, Y., Barker, L., Lincoln, D., Crumley, K.V., Franki, J.E., Schechter, N.M., Lazarus, G.G., Nakajima, K., Nakashino, K., Neurath, H. and Woodbury, R.G. (1985) Biochemistry 24, 2048-2058

Pozsgay, M., Gáspár, R., Bajusz, S. and Elődi, P. (1979) Eur. J. Biochem. 95, 115-119

Pozsgay, M., Cs.-Szabó, G., Bajusz, S., Simonsson, R., Gáspár, R. and Elődi, P. (1981) Eur. J. Biochem. 115, 491-495

Pozsgay, M., Cs.-Szabó. G., Bajusz, S., Simonsson, R., Gáspár, R. and Elődi, P. (1981) Eur. J. Biochem. 115, 497-502

Reilly, C.F. and Travis, J. (1980) Biochim. Biophys. Acta 621, 147-157

Schechter, I. and Berger, A. (1967) Biochim. Biophys. Res. Commun. 27, 157-162

Schmidt, W. and Havemann, K. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1077-1082

Starkey, P.M., Barrett, A.J. and Burleight, M.C. (1977) Biochim. Biophys. Acta 483, 386-397

Tanaka, T., Minematsu, Y., Reilly, C.F., Travis, J. and Powers, J.C. (1985) Biochemistry 24, 2040-2047

Tőzsér, J., Cs.-Szabó, G., Pozsgay, M., Aurell, L. and Elődi, P. (1986) Acta Biochim. Biophys. Hung. 21, 335-348

Wilkinson, G.N. (1961) Biochem. J. 80, 324-332

Yoshida, N., Everitt, M.J., Neurath, H., Woodbury, R.G. and Powers, J.C. (1980) Biochemistry 19, 5799-5804

Zimmerman, M. and Ashe, B.M. (1977) Biochim. Biophys. Acta 480, 241-245

Acta Biochim. Biophys. Hung. 21(4), 363-367 (1986)

CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY RELATIONSHIP IN BRADYKININ ANALOGUES CONTAINING PROLINE-LIKE UNUSUAL AMINO ACIDS

L. Baláspiri, M.V. Tóth, J. Lonovics[*], K. KOvács

Institute of Medical Chemistry and 1st Department of Medicine[*], University Medical School of Szeged, Szeged, Hungary

(Received January 15, 1984)

SUMMARY

Ten different bradykinin analogues have been synthesized and tested for activity on isolated guinea-pig ileum. The results, in comparison with others in the literature, suggest some conclusions on a chemical structure - biological activity relationship.

INTRODUCTION

Slight changes in the structure of known peptide hormones, by substituting amino acids of similar character for constituent ones, are very important in the investigation of the relationship between chemical structure and biological activity (Schröder and Lübke, 1966; Rudinger, 1971). A great development from this respect was achieved about fifteen years ago, when synthetic, optically active unusual amino acids were first applied in peptide chemistry to synthesize different structural analogues of known peptide hormones (Rudinger, 1971; Sheppard, 1969-1980). Our peptide group was the first to apply some proline-like unusual amino acids (Kovács et al., 1971).

Optically active L- and D-pipecolic acid (Baláspiri et al., 1970) and also L- and β-D-homoproline (pyrrolidine-2-acetic

Abbreviations: AVP, 8-arginine-vasopressin; HPro, β-L-homoproline = pyrrolidine-2-acetic acid; MIF, melanotropin inhibiting factor; Pip, pipecolic acid; TRH, thyrotrop releasing hormone

Akadémiai Kiadó, Budapest

acid) (Baláspiri et al., 1975) have been used for the synthesis of bradykinin analogues (Baláspiri et al., 1975; Neubert et al., 1972) containing originally three proline residues. Numerous papers can now be found reporting the application of proline-like optically active pipecolic acid or β-homoproline. Analogues of collagen models (Katchalski et al., 1963), oxytocin (Bespalova et al., 1966), angiotensin II (Chaturvedi et al., 1970), sequence-polypeptide (Fairweather and Jones, 1972), TRH (Bankowski and Drabarek, 1976) and model peptides (Vicar et al., 1973a; 1973b) containing pipecolic acid have been reported. During the past few years, not only bradykinin analogues (Baláspiri et al., 1975; Neubert et al., 1972) but also other similar analogues of TRH, AVP (V. Toth, 1983), pentagastrin, kyotorphin (Baláspiri et al., 1983), β-casomorphine-5 (Hartrodt et al., 1986), MIF (Baláspiri, L., Institoris, L., Kovács, G., Szabó, Gy., Telegdy, Gy., Kovács, K., 1986; unpublished observation) and morphine dependence, tolerance inhibiting peptide (Baláspiri et al., 1983) containing L- or D-pipecolic acids have been synthesized by our research group.

Only few reports applying β-L- or β-D-homoproline for the synthesis of peptide analogues can be found in the literature. One of our bradykinin analogues was also synthesized by Ondetti and Engel (1975) and MIF analogue (Baláspiri et al., 1986; unpublished observation) by Bankowski and Misicka (1983) without synthetic details or biological data. Similar analogues of kyotorphin (Baláspiri et al., 1983), AVP (V. Toth, 1983), pentagastrin and others have been synthesized and are undergoing biological examinations, and have therefore not been published so far.

RESULTS AND DISCUSSION

Our synthetic bradykinin derivative 1-^{14}C-labelled in the glycine residue, and ten analogues containing L- or D-pipecolic acid or β-L-homoproline, were tested on isolated guinea-pig ileum for agonist activity; this is the most commun system for this aim (Wiepershausen et al., 1964). The results were com-

pared with those for standard bradykinin (given as percentages in Table 1).

Table 1. Biological activity of bradykinin analogues

Analogues	Biological activity
BRADYKININ	100%
2-L^{x}-Pip-Bradykinin	25%
7-L-Pip-Bradykinin	60%
2, 3-L-Pip-Bradykinin	10%
2, 7-L-Pip-Bradykinin	40%
3, 7-L-Pip-Bradykinin	30%
2, 3, 7-L-Pip-Bradykinin	inactive
2-D-Pip-Bradykinin	inactive
3-D-Pip-Bradykinin	inactive
7-D$_{xx}$-Pip-Bradykinin	inactive
7-β-L-HPro-Bradykinin	100%
7-β-L-HPro-Bradykinin	100%
^{14}C-Bradykinin	100%

The biological results in the common test indicate that we successfully synthesized bradykinin, ^{14}C-bradykinin with 370 MBq/mmol specific activity and the listed analogues. The bradykinin (Arg-Pro-Pro-Gyl-Phe-Ser-Pro-Phe-Arg) contains three Pro residues in positions 2, 3 and 7. An interesting question for us was how the rigid bradykinin molecule and its biological activity are influenced if it contains optically active proline-like unusual amino acids. This provides information about the chemical structure and biological activity relationship of the peptide hormone.

We can conclude that not too drastic change, a slightly larger ring or a longer side-chain with the same configuration, in position 7 do not decrease the activity sensitively. Posi-

tion 2 is more sensitive, and position 3 is the most sensitive towards the same changes. This is proved in the double or triple-substituted analogues. Changes in configuration of amino acids in any position are undesirable as concerns the agonist activity of bradykinin. The activity is then totally lost, the configurational change influences the molecular conformation of bradykinin, inhibiting the binding at its receptor site. Similar conclusions can be found in the literature (Schroder and Lübke, 1966; Sheppard, 1969-80).

We plan to obtain more evidence for this theory. Similar analogues are therefore being synthesized and tested, and conformational examinations are under way.

REFERENCES

Baláspiri, L., Penke, B., Petres, J., Kovács, K. (1970) Monatshefte 101, 1177

Baláspiri, L., Penke, B., Papp, Gy., Dombi, Gy., Kovács, K. (1975) Helv. Chim. Acta 58, 969

Baláspiri, L., Papp, Gy., V. Toth, M., Sirokmán, F., Kovács, K. (1979) 25, 179

Baláspiri, L., Neubert, K., Ottlecz, A., Gecse, Á., Kovács, K., Telegdy, Gy. (1983) Proc. 17th Eur. Peptide Symp. (K. Blaha and P. Malon, eds.), Walter de Gruyter, Berlin, 487-493

Bankowski, K., Drabarek, S. (1976) Acta Polon. Pharm. 33, 81

Bankowski, K., Misicka, A. (1983) Proc. 17th Eur. Peptide Symp. (K. Blaha and P. Malon, eds.) Walter de Gruyter, Berlin, 331-332

Bespalova, Z.D., Kairov, O.A., Martinov, U.P., Natosky, V.U., Titov, M.I., Sachamtova, E.I. (1966) Vest. Leningrad Univ. Ser. Fiz. Khim. 21, 157

Chaturvedi, N.C., Park, W.K., Smeby, R.R., Bumpus, F.M. (1970) J. Med. Chem. 13, 177

Fairweather, R., Jones, J.H. (1972) J. Chem. Soc. Perkin Trans. Vol. I, 2475

Hartrodt, B., Berger, E., Neubert, K., Baláspiri, L. (1986) Pharmazie (in press)

Katchalski, E., Berger, A., Kurtz, J. (1963) Symp. Protein Structure and Crystallography, Madras

Kovács, K., Penke, B., Czombos, J., Petres, J., Baláspiri, L. (1971) Acta Phys. Szeged, 17, 61

Neubert, K., Baláspiri, L., Losse, G. (1972) Monatshefte 103, 1573

Ondetti, M.A., Engel, S.L. (1975) J. Med. Chem. 18, 761

Rudinger, J. (1971) in "Drug Design", Vol. 1, Acad. Press, New York-London

Schröder, E., Lübke, K. (1966) in The Peptides, Vol. 2, Acad. Press, New York-London

Sheppard, B. (1969-1980) "Amino-acids, Peptides and Proteins" Special Periodical Report, The Chemical Society, London, Vol. 1-12

V. Toth, M. (1983) Ph.D. Thesis, Szeged

Vicar, J., Smolikova, J., Blaha, K. (1973a) Coll. Czechosl. Chem. Commun. 38, 1940

Vicar, J., Smolikova, J., Blaha, K. (1973b) Coll. Czechosl. Chem. Commun. 38, 1967

Wiepershausen, B., Stopp, G. and Eichstadt, M. (1964) Acta Biol. Med. Ger. 12, 443

Acta Biochim. Biophys. Hung. 21/4/, pp.369-379 /1986/

TEMPERATURE-INDUCED SPECTRAL PROPERTIES OF CHLOROPHYLL a INCORPORATED INTO EGG-YOLK LECITHIN LIPOSOMES AND LIPO-PROTEIN COMPLEXES

G.E. Bialek-Bylka, D. Wróbel

Institute of Physics, Poznan Technical University, Poznan, Poland

(Received November 25, 1985)

The temperature-induced fluorescence changes of Chl a in the systems consisting of albumin-egg-yolk lecithin and only lecithin were studied. As plotted against temperature in liposomes the fluorescence intensity of Chl a has a maximum at approximately 35 ^{o}C. In CLP complexes the curve profile for the fluorescence intensity versus temperature is sharper and the maximum occurred at a lower temperature (25 ^{o}C). No fluorescence maxima for ANS in liposomes and for Chl a in isotropic solutions were found. The second derivative absorption spectra of the investigated lipid systems exhibited bands which were characteristic of monomeric Chl a.

We suggested that in the temperature zone studied Chl a in vitro is embedded within lipid bilayer rather than attached to the proteins. The occurrance of the fluorescence intensity maximum of Chl a in liposomes and lipo-protein complexes is related to the effects: 1/ temperature quenching of the fluorescence, 2/ Chl a-lipid interaction.

INTRODUCTION

Photosynthetic reactions take place in the lipoprotein matrix of the chloroplast membranes (Eigenberg et al., 1981; Hubbell, Connell, 1971; Lichtenthaler, Park, 1963; Lutz, 1977; Luzzati, Husson, 1962; Tien, 1967; Vanderkooi, 1974). The amphiphatic phospholipids in bilayer form a smectic structure, i.e. a two-dimensional liquid crystal (Luzzati, Husson, 1962; Tien, 1967; Allen, Good, 1971; Harwood, Stumpf, 1969). The physical state

Abbreviations: Chl a - Chlorophyll a
CLP - Chlorophyll a-Lipo-Protein
ANS - 1 anilino - 8 naphtalene sulfonate
HSA - Human Serum Albumin

Akadémiai Kiadó, Budapest

of the lipids (Cogan et al., 1973; Feinstein et al., 1975; Lee, 1975a; Lee, 1975b; Warren et al., 1974; Warren et al., 1975) plays an important role both in the photosynthetic reactions (Murata, Fork, 1975a; Murata et al., 1975; Shneyour et al., 1973), and in the physiological activities (Kemp et al., 1969; Lyons et al, 1964; Lyons, Raison, 1970; Overath et al., 1970; Raison et al., 1971a; Trauble, Eibl, 1974) and functions of the membranes (Vanderkooi, 1974; Overath, Trauble, 1973; Raison et al., 1971b). According to Hoshina et al. (Hoshina et al., 1984), the presence of lipids is essential, in order to see a clear maximum in the fluorescence versus temperature curve of Chl a in a chl-protein complex. It is suggested that in vivo a specific form of Chl a is responsible for the temperature - induced absorbance and fluorescence yield changes (Hoshina et al., 1984).

The main photosynthetic pigment in these membranes, i.e. the Chl a molecule, is composed of a porphyrin ring which contains a hydrophilic and a hydrophobic region and a strongly hydrophobic phytol chain. The thermodynamically most stable condition of the Chl a molecule in the lipid bilayer is achieved when the phytol is associated with the hydrocarbon region of the membrane lipids and the porphyrin ring is situated at the surface of the membrane (Murata, Fork, 1975a; Cherry et al., 1972a; Cherry et al., 1972b; Hoff, 1974; Oettmeier et al., 1976a; Podo et al., 1976; Steinemann et al., 1972). Many forms of Chl a in vivo can be produced by chl - chl (Katz et al., 1976), chl - lipid (Hoshina, 1981) or chl - protein (Markwell et al., 1979; Sugiyama, Murata, 1978) interactions.

In model membranes the fluorescent probes show maximum fluorescence intensities at the transition between the mixed solid/liquid crystalline and the pure liquid crystalline states. On the other hand, a minimum of the fluorescence intensity appears when lipids move from the solid to the mixed solid/liquid crystal phase (Murata, Fork, 1975a; Overath, Trauble, 1973; Colbow, 1973; Sackmann et al., 1973). In lipid systems containing water the lyotropic mesomorphic change from one liquid crystalline phase to another is observed at temperatures above that of solid/liquid crystal transition (Overath, Trauble, 1973). The hydration capacity of the membrane depends on the phase state of lipids and the structure as well as on the other molecules incorporated into the membrane. Since lyotropic phases also exhibit thermotropic mesomorphism, any particular phase is a function of both water content and temperature.

According to Ladbrooke and Chapman (Ladbrooke, Chapman, 1969), the phase transition temperature from solid to liquid crystalline state of egg-yolk lecithin is at about minus 10°C. There is little information either on the Chl a behaviour in artificial lipid membranes or on the Chl a influence on the physical state of lipids over a range of the physiological temperatures (0-50°C). Moreover, although some reliable data concerning the phase of lipids have been reported (Cogan et al., 1973; Feinstein et al., 1975; Lee, 1975a; Warren et al., 1974; Warren et al., 1975), still little is known about the proteins in the model membranes (Feinstein et al., 1975; Quinn, 1981).

Therefore, the temperature dependence of the spectral properties of Chl a was investigated by fluorescent probes in liposomes and CLP complexes model systems (Strauss, 1976; Tombacz et al., 1982).

MATERIALS AND METHODS

Chl a was prepared chromatographically (Iriyama et al., 1974). The concentration was estimated from the absorption spectrum, using the absorption coefficient given by Strain and Svec (Strain, Svec, 1966). CLP complexes and liposomes were obtained according to the method proposed by Tombacz et al. (1982). The stock concentration of Chl a was 0.01 g/ml and the molar ratio of Chl a to lecithin was 0.5. Egg-yolk lecithin (Merk FRG) and HSA (Reanal Budapest, Hungary) were used. The fluorescence spectra were recorded with a device described earlier (Frackowiak et al., 1977). The samples containing Chl a and ANS were excited at wavelengths of 436 nm and 360 nm, respectively. Emission spectra of Chl a (650-800 nm) and ANS (460-650 nm) at temperature zones of 1.5-50°C were measured. The band width for either the exciting or the analysing beam was not exceeding 6 nm. Second derivative absorption spectra were recorded by a Hitachi 356 spectrophotometer with 2 nm differentiating interval.

RESULTS

Fig. 1 a and b show the normalized (at the maximum of the ratio F/F_0; where F and F_0 are the intensities of the maxima of the fluorescence spectra at the given and the lowest temperature, respectively) temperature

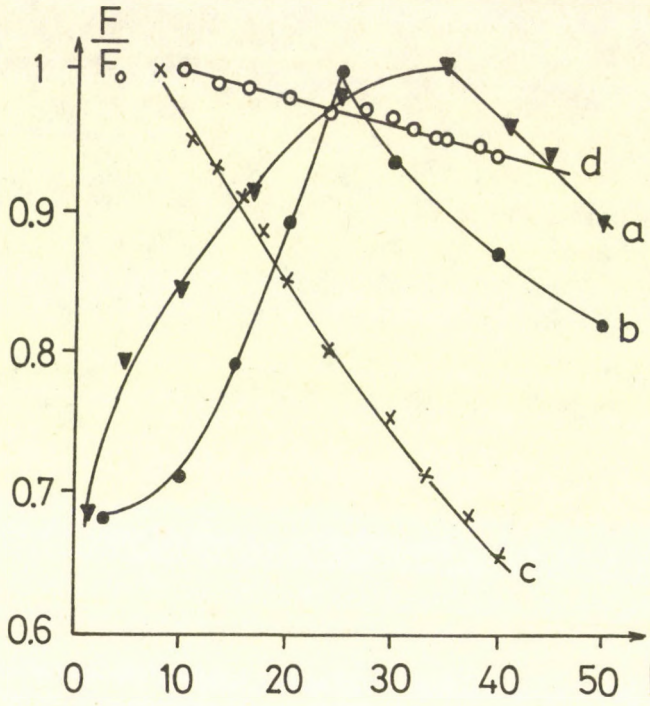


Fig. 1 Fluorescence - temperature dependence curves: a, b, c, d of: Chl a - liposomes, CLP complexes, Chl a in ethyl ether and ANS - liposomes, respectively, (F, F_0 and normalization - see result section).spectra in liposomes and CLP complexes are

dependence of the fluorescence intensities of Chl a in liposomes observed at 683 nm and in CLP complexes at 680 nm. The fluorescence intensity of Chl a in the two systems increases as a result of heating over the temperature range of 1.5-35 ^{o}C for liposomes and in the range of 1.5-25 ^{o}C for the complexes. This increase is steeper in the CLP complexes than in liposomes. No minima were found in the measured temperature zones in either samples. Comparing the results of CLP complexes and liposomes, one observes a protein-induced shift of the fluorescence intensity maximum on the temperature scale and a narrowing of curve b in Fig. 1. The fluorescence maximum of Chl a in CLP complexes is "blue" shifted, with respect to that in the liposomes (data not demonstrated here).

The curves c and d in Fig. 1 represent the fluorescence intensities of Chl a in ethyl ether solution and ANS in liposomes, respectively. They show a gradual and monotonic decrease with increasing temperature, in

accordance with the data of other authors (Murata, Fork, 1975b; Biaselle, Millar, 1975). The second derivative absorption spectra of liposomes and CLP complexes at room and liquid nitrogen temperatures are displayed in Fig. 2. The low temperature "red" peaks of the Clh a derivative absorption spectra in liposomes and CLP complexes are located at the same position (669 nm) but the room temperature absorption maximum in liposomes is shifted 4 nm to the shorter wavelength compared with that of CLP complexes. At low temperatures the Soret band maxima of both the liposomes and the CLP complexes are shifted to longer wavelengths, as compared with those at room temperature. The liposome absorption Soret band peaks are blue shifted (3nm at low temperature and 4 nm at room temperature), as compared with the corresponding peaks of CLP complexes.

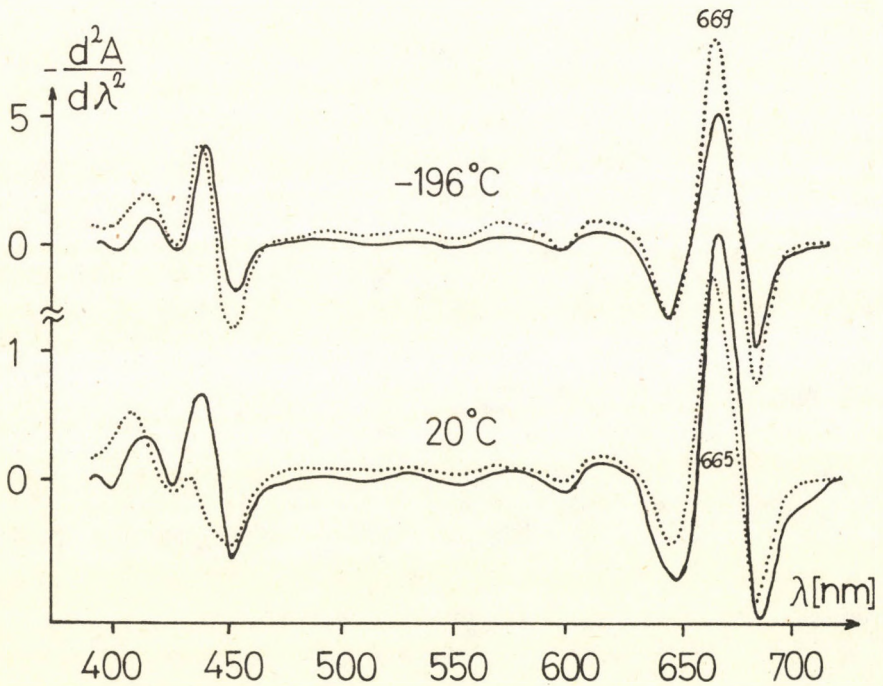


Fig. 2 Second derivative of absorption spectra of: Chl a - liposomes (·····) and CLP complexes (-·---). For both samples measurement temperatures were (-196 °C) and 20 °C.

DISCUSSION

The sharpness of a phase transition corresponds to the size of so--called cooperative unit-number of molecules forced to cooperate in the transition. A "sharp" transition, i.e. a first-order phase transition, occurs in a perfect infinite crystal only. It has been shown (Hinz, Sturtevant, 1972) that in lipid bilayers the cooperative unit is quite small, i.e. the melting does not take place directly from a crystalline to a liquid state in a cooperative manner. In such systems transition has both first- and second-order characteristics (Mitaku et al., 1983). Our results show a characteristic temperature dependence of Chl a fluorescence intensity both in liposomes and in CLP complexes (Fig. 1 a and b). This effect can be related to the temperature - induced spectral properties of Chl a - lipid systems. The absence of the fluorescence intensity minimum for both liposomes and CLP complexes (Fig. 1) in the temperature range investigated suggests that the lipids exist either in the liquid or in the mixed solid/liquid state but not in the solid state. There is no evidence in the literature to indicate that the Chl molecules are preferentially situated into the solid region of the lipid membrane. Thus we may presume a uniform distribution of Chl a throughout the liposomes (Fork et al., 1981). As a result, the curve of the temperature-induced fluorescence intensity changes is rather broad when Chl is used as the fluorescent probe. An additional reason for the observed effect can be the presence of water. Phospholipids do not pass directly from the crystalline state to a solution, but proceed through certain well defined hydrated phases (Penkett et al., 1968). Therefore, observing a bread curve of the thermal changes of the fluorescence intensity of Chl a - liposomes is not unexpected.

In the solid state the number of bound probe molecules is reduced, hence weak fluorescence emission is observed (Overath, Trauble, 1973). The rate of internal conversion is decreased and, consequently, both the quantum yield and the lifetime will increase by restrictions on the vibrational or rotational freedom of the excited molecules (Strauss, 1976). For instance, this can happen when a molecule becomes bound to a membrane. On the basis of the concept of Feinstein et al. (1975), in lipid systems the fluorescence lifetime varies with temperature, similarly to the change in the fluorescence intensity. We suggest that in the temperature-induced maxima (Fig. 1 a and b) two effects are superimposed: 1/ temperature

quenching of the fluorescence, and 2/ Chl a - lipid interactions (Murata, Fork, 1975a). This conclusion is supported by the experiments with Chl a ethyl ether solution (Fig. 1 c) and ANS liposomes (Fig. 1 d). Under isotropic condition (Chl a in ethyl ether) the monotonic decay of fluorescence intensity with increasing temperature is mainly due to collision quenching. ANS is a fluorescent probe which is bound to the polar outer side of the lipid membrane (Vanderkooi, 1974; Badley et al., 1973; Sackmann, Trauble, 1972; Raada, Vanderkooi, 1972). For the Chl a - lecithin system one would obtain a temperature dependence for the fluorescence, similar to that of the ANS - lipid system, if there would be no specific interaction between Chl a and lecithin. In the solid membrane, the porphyrin ring of Chl a is attached to the membrane surface much resembling to ANS. In this case Chl a is very weakly fluorescent (Murata, Fork, 1975b). When temperature is being increased, and more and more porphyrin rings penetrate the hydrocarbon region of the membrane, the Chl a fluorescence yield increases. With increasing temperature the anisotropic distribution of Chl a in the lecithin membrane becomes more isotropic and the Chl a molecules behave like in isotropic solutions (ether, for instance). Neglecting temperature quenching, the curve of the fluorescence intensity versus temperature should reach a constant level due to the quasi-isotropic distribution of Chl a molecules in the membrane interior. However, for temperatures higher than 35°C for liposomes and 25°C for CLP complexes the fluorescence intensity curves of these systems are similar in character to the monotonically decreasing curve of Chl a in ethyl ether. This indicates that above these temperatures the Chl a - lipid systems are almost isotropic and the temperature quenching effect begins to play a dominant role. The hydration of a phospholipid dispersion increases significantly with temperature because the bilayer surface expands as a result of the increasing volume and because of isomer formation and a simultaneous increase in the mobility of the lipid molecules (Overath, Trauble, 1973; Taylor et al., 1978; Watt et al., 1978). As a consequence, more water molecules and particles enter the bilayer and interact with the polar head groups (Penkett et al., 1968; Taylor et al., 1978; Watt et al., 1978).

An increase in ionic strength reduces the temperature dependence due to the decrease in electrostatic repulsion between negatively charged lipid molecules (Overath, Trauble, 1973). The electrostatic potential created by

the phospholipid layers depends strongly on the conformation of the constituent molecules, particularly of their polar heads, as well as on the organization of the molecules in the layers (Zakrzewska, Pulman, 1981).

In the bilayers there are globular proteins which are of a great functional variety (Vanderkooi, 1974). These so-called intrinsic proteins are located in different depths of the lipid bilayers. The albumin is a transport protein and therefore it can affect the membrane hydration and polarity. The blue shift of the fluorescence maximum of Chl a in CLP complexes results in the transfer of the fluorescence probe to the less polar solvent (Brand, Gohlke, 1972). The incorporation of the albumin causes the perturbation of the molecular arrangement of lipids from a relatively ordered state ot a more disordered one (Nagle, Wilkinson, 1978; Gruen, 1980). Thus, for the CLP complexes the rise of the fluorescence intensity versus temperature is found to be more abrupt than for liposomes. The nature of the interaction between fluorescence probes and solvent molecules affects also the position of absorption bands. The chlorophyll, because of its electrophile character, is always complexed either with a solvent molecule or with another chlorophyll. The second derivative absorption spectra of liposomes and complexes used here (Fig. 2) exhibit at liquid nitrogen temperature red absorption band with a maximum at 669 nm which is characteristic of monomeric Chl a (Oettmeier et al. 1976b). According to Murata and Sato (1978), the Chl a-670 form is a product of the interaction of Chl a with lipid in an aqueous dispersion. An aggregation of Chl incorporated into the lipid bilayer is weaker when the lipids are in the liquid state than when they are in the gel sate (Lee, 1975a). Formation of non-fluorescent aggregates is ruled out by the absence of a long-wavelength absorption band. Comparing the second derivative absorption curves of CLP complexes obtained low (liquid nitrogen) and room temperatures, no red shift is observed, which is in accordance with the data of Leclero et al (1976) and shows that there is no effect of the albumin on the rigid lipid system. The blue shift of the room temperature second derivative absorption maxima of Chl a-liposomes, with respect to that at low temperature, indicates that in a membrane solute the pigments can be in a so-called card pack arrangement. Moreover, the relative shift of the "red" absorption maxima of Chl a-liposomes and CLP complexes marks the polarity increase of the Chl a surroundings in the lipid membrane.

Finally, we assumed that in vitro the chlorophyll is embedded within the lipid bilayer rather than attached to the ANS (Eigenberg et al., 1981; Anderson, 1975). The albumin incorporated into Chl - lecithin liposomes induces phase transition at lower temperature than Chl - lecithin liposomes alone. The lipid membranes inhibit quenching of the fluorescence of chlorophyll a.

Our data suit well for the results of Hoshina (1981) and Murata et al. (Murata, Fork, 1975a; Murata et al., 1975), although they do not support the concept of Markwell et al. (1979) that in vivo all chlorophyll exists in the form of chlorophyll-protein complexes.

Acknowledgements

The authors thank Professor D. Frackowiak for suggestions and discussion. The spectra with the Hitachi spectrophotometer were obtained in the Institute of Photosynthesis of the USSR Academy of Sciences in Pushchino.

This study was carried out under Project No MR II.7.

REFERENCES

Allen, C.F., Good, P. (1971) Methods Enzymol. 23 523-547

Anderson, J.N. (1975) Nature 253 536-537

Badley, R.A., Martin, W.G., Schneider, H. (1973) Biochemistry 12 268-275

Biaselle, C.J., Millar, D.B. (1975) Biophys. Chem. 3 355-361

Brand, L., Gohlke, J.R. (1972) Ann. Rev. Biochem. 41 843-868

Cherry, R.J., Hsu, K., Chapman, D. (1972a) Biochim. Biophys. Acta 267 512-522

Cherry, R.J., Hsu, K., Chapman, D. (1972b) Biochim. Biophys. Acta 288 12-21

Cogan, U., Shinitzky, M., Weber, G., Nishida, T. (1973) Biochemistry 12 521-528

Colbow, K. (1973) Biochim. Biophys. Acta 318 4-9

Eigenberg, K.E., Groasmun, W.R., Chant, S.I. (1981) Biochim. Biophys. Acta 642 438-442

Feinstein, M.B., Fernandez, S.M., Sháafi, R.I. (1975) Biochim. Biophys. Acta 413 354-370

Fork, D.C., Van Ginkel, G., Harvey, G. (1981) Plant Cell Physiol. 22 1035-1042

Frackowiak, D., Bauman, D., Manikowski, H., Martynski, T. (1977) Biophys. Chem. 6 369-377

Gruen, D.W.R. (1980) Biochim. Biophys. Acta 595 161-183

Harwood, J.L., Stumpf, P.K. (1969) TIBS 1 253-256

Hinz, H.J., Sturtevant, J.M. (1972) J. Biol. Chem. 247 3694-3697

Hoff, A.J. (1974) Photochem. Photobiol. 19 51-57

Hoshina, S. (1981) Biochim. Biophys. Acta 638 334-340

Hoshina, S., Mohanty, P., Fork, D.C. (1984) Photosynth. Res. 5 347-360

Hubbell, W.L., McConnell, H.M. (1971) J. Am. Chem. Soc. 93 314-326

Iriyama, K., Ogura, N., Takamiya, A. (1974) J. Biochem. 76 901-904

Katz, J.J., Oettmeier, W., Norris, J.R. (1976) Phil. Trans. Roy. Soc. Lond. B 273 227-253

Kemp, A.Jr., Groot, G.S.P., Reitsma, H.J. (1969) Biochim. Biophys. Acta 180 28-34

Ladbrooke, B.D., Chapman, D. (1969) Chem. Phys. Lipids 3 304-367

Leclerc, J.C., Hoaran, J., Remy, R. (1976) Biochim. Biophys. Acta 547 398-409

Lee, A.G. (1975a) Biochim. Biophys. Acta 413 11-23

Lee, A.G. (1975b) Biochemistry 14 4397-4402

Lichtenthaler, H.K., Park, R. B. (1963) Nature 198 1070-1072

Lutz, M. (1977) Biochim. Biophys. Acta 460 408-430

Luzzati, V., Hussen, F. (1962) J. Cell Biol. 12 207-219

Lyons, J.M., Wheaton, T.A., Pratt, H.K. (1964) Plant Physiol. 39 262-268

Lyons, J.M., Raison, J.K. (1970) Plant Physiol. 45 386-389

Markwell, J.P., Thornber, J.P., Boggs, R.T. (1979) Proc. Natl. Acad. Sci. USA 76 1233-1235

Mitaku, S., Jippo, T., Kataoka, R. (1983) Biophys. J. 42 137-144

Murata, N., Fork, D.C. (1975 a) Plant Physiol. 56 508-517

Murata, N., Fork, D.C. (1975 b) Plant Physiol. 56 791-796

Murata, N., Sato, N. (1978) Plant Cell Physiol. 19 401-410

Murata, N., Troughton, J.M., Fork, D.C. (1975) Plant Physiol. 56 508-517

Nagle, J.F., Wilkinson, D.A. (1978) Biophys. J. 23 159-175

Oettmeier, W., Norris, J.R., Katz, J.J. (1976a) Biochem. Biophys. Res. Comm 71 445-451

Oettmeier, W., Norris, J.R., Katz, J.j. (1976b) Z. Naturforsch. 310 163-168

Overath, P., Schairer, H.U., Stoffel, W. (1970) Proc. Natl. Acad. Sci. USA 67 606-612

Overath, P., Trauble, H. (1973) Biochemistry 12 2625-2634

Penkett, S.A., Flook, A.G., Chapman, D. (1968) Chem. Phys. Lipids 2 273-290

Podo, F., Cain, J.E., Blasie, J.K. (1976) Biochim Biophys. Acta 419 19-41

Quinn, P.J. (1981) Prog. Biophys. Mol. Biol. 38 1-104

Raada, G.K., Vanderkooi, J.V. (1972) Biochim. Biophys. Acta 265 509-549

Raison, J.K., Lyons, J.M., Thompson, W.W. (1971 a) Arch. Biochem. Biophys. 142 83-90

Raison, J.K., Lyons, J.M., Mehlhorn, R.J., Keith, A.D. (1971b) J. Biol. Chem. 246 4036-4040

Sackmann, E., Trauble, H. (1972) J. Am. Chem. Soc. 94 4499-4510

Sackmann, E., Trauble, H., Galla, H.J., Overath, P. (1973) Biochemistry 12 5360-5369

Shneyour, A., Raison, J.K., Smilke, R. (1973) Biochim. Biophys. Acta 292 152-161

Steinemann, A., Stark, G., Leugen, P. (1972) J. Membr. Biol. 9 177-194

Strain, H.H., Svec, W.A. (1966) in: The Chlorophylls. (eds.: Vernon, L.R., Seely, G.R.), Academic Press, New York-London, p. 21-66

Strauss, G. (1976) Photochem. Photobiol. 24 141-153

Sugiyama, K., Murata, N. (1978) Biochim. Biophys. Acta 503 107-119

Taylor, R.P., Hang, G., Broccoli, A.V., Chun, J.K. (1978) Arch. Biochem. Biophys. 187 197-200

Tien, H.T. (1967) J. Theoret. Biol. 16 97-110

Tombácz, E., Várkonyi, Z., Szalay, L. (1982) Zh. Prikl. Spektr. 36 64-72

Trauble, H., Eibl, H. (1974) Proc. Natl. Acad. Sci. USA 71 214-219

Vanderkooi, G. (1974) Biochim. Biophys. Acta 344 307-345

Warren, G.B., Tcon, P.A., Birdsall, N.J.M., Lee, A.G., Metcalfe, J.C. (1974) Biochemistry 13 5501-5507

Warren, G.B., Metcalfe, J.C., Lee, A.G., Birdsall, N.J.M. (1975) FEBS Lett. 501 261-264

Watt, S., Harsh, D., Knowles, P.F. (1978) Biochemistry 17 1792-1801

Weller, H.G., Tien, H.T. (1973) Biochim. Biophys. Acta 325 433-440

Zakrzewska, K., Pulman, B. (1981) FEBS Lett. 131 77-80

Acta Biochim. Biophys. Hung. 21/4/, pp. 381-390 /1986/

VARIATIONS IN ACETYLCHOLINESTERASE FROM BRAIN AND MUSCLE OF A FRESHWATER AIR-BREATHING TELEOST, HETEROPNEUSTES FOSSILIS

B.K. Ratha, S.N. Ramanujan

Biochemical Adaptation Laboratory; Department of Zoology;
North-Eastern Hill University; Shillong - 793 014; India

(Received August 25, 1986)

Specific staining on polyacrylamide gel of acetylcholinesterase (AchE) from brain and muscle of **Heteropneustes fossilis** showed one major tissue specific band in each. Eserine inhibited the enzyme competitively in both tissue homogenates. However, the normal level of activity and the pattern of the rate of inhibition with increasing eserine concentrations were different. Muscle showed higher AchE specific activity than brain. There was a hyperbolic increase in the percent of inhibition of AchE activity by eserine in muscle whereas in brain the pattern was biphasic. The apparent Km and Vmax in the two tissue homogenates were also different. The results suggest structural and functional variations of AchE in brain and muscle of **H. fossilis**.

INTRODUCTION

Many enzymes have been reported to exist in multiple molecular forms or as isoenzymes showing variations in their structure, synthesis, distribution and biochemical functions of physiological significance (Markert, 1968). They have been used as excellent models to study gene regulation by different physico-chemical modulators and to study biochemical differentiation during development (Rider, Taylor, 1980). Acetylcholinesterase (AchE;EC.3.1.1.8), an important enzyme in neurotransmission, has been reported to be present in cholinergic nerve synapses (Nachmanshon, 1959), neuro-muscular junctions (Hall, 1973) and nerve free end-plates (Weinberg, Hall, 1979). It has been shown to exist in multiple molecular forms. The variations in the levels of AchE activity, its

isoenzyme pattern, kinetics and response to different physiological modulators have been correlated with the functional adaptations of different tissues in several vertebrates (Bernsohn, et al., 1963; Maynard, 1964; Baldwin, Hochachka, 1970; Baldwin, 1971; Moudgil, Kanungo, 1973; Vijayan, Brownson, 1975; Vijaylakshmi, et al., 1977; Gisieger et al, 1978; Principato, et al., 1978; Ramanujam, Ratha, 1981; 1983). Isoenzymic variations have been reported during early embryonic development in different species and during muscle differentiation and dystrophy in higher vertebrates (Whittaker, 1979; Fluck, 1978; Michalek et al., 1984; Lappin, Rubin, 1985). Detailed studies on structural and functional variations have been done in AchE purified from the brain of some higher vertebrates and from the electric organ of **Torpedo** and **Electricus** (Vigny et al., 1976; 1979; Bon et al., 1976; Rieger et al., 1976; Carson et al., 1979). There have been fragmentary reports on tissue specific variations of AchE in freshwater teleosts, particularly airbreathing teleosts. We have earlier reported variations in the physiological level (Principato et al., 1978) and the inhibition by a phyto-toxin (Ramanujam, Ratha, 1981) of AchE activity in brain and muscle tissues of some freshwater teleosts. The reports indicated that AchE in the two tissues studied could be both structurally and functionally different. This paper presents our finding on different molecular forms observed by specific staining on polyacrylamide gels and the kinetic variations of AchE from brain and muscle of a fresh water air-breathing teleost, **Heteropneustes fossilis**.

MATERIALS AND METHODS

The air-breathing fish **H. fossilis** weighing between 12-15g were purchased from local suppliers. They were maintained in the laboratory for three weeks at 20±2°C in plastic aquaria containing tap water. They were fed with dry fish powder on alternate days. The fishes were sacrificed at a fixed time of the day by decapitation and the tissues (brain and muscle) were removed immediately, washed in ice-cold 0.25 M sucrose and blotted dry. A 5% homogenate of each tissue was prepared in ice-cold 0.25 M sucrose using an all glass Potter-Elvehjem type homogeniser. The homogenate was centrifuged at 14000 x g at 0±2°C for 20 minutes and the supernatant was used for the estimations. AchE activity was assayed spectrophotometrically following the method of Ellman et al (1961) using acetylthiocholine iodide

as the substrate. The protein concentration in the supernatant was estimated following Lowry et al (1951) using bovine serum albumin as the standard. The activity of AchE of brain and muscle were assayed at 1, 2, 3, 4, 5, 8, 10 and 20 x 10^{-5}M concentrations of the substrate to determine the Km and Vmax by Lineweaver-Burk plot. The percent and pattern of inhibition of enzyme activity and the nature of inhibition by eserine with acetylthiocholine iodide as substrate were also determined.

Polyacrylamide gels were prepared following Davis (1964). Electrophoresis was carried out using 7.5 per cent gels and with a constant current of 4 mA per gels for 2 hours at 2 $^{+}$ 2^{o}C using 0.02 M tris-citrate buffer at pH 8.6 (Baldwin, Hochachka, 1970). Duplicate gels were run concurrently. One set was specifically stained for AchE activity following the method of Koelle (1951) and the other set was treated with eserine before staining for AchE activity to find out specific AchE bands. The gels were then scanned densitometrically at 425 nm in a scanning spectrophotometer (Beckman model-26).

All chemicals used were of analytical grade purchased from either Sigma Chemical Co., U.S.A., Glaxo Laboratories or SISCO Laboratories, India. Double glass distilled water was used for all preparations.

RESULTS

The specific activity of AchE was different in brain and muscle of **H. fossilis** (Table 1). Muscle had a relatively higher specific activity than brain. The inhibition by eserine also showed independent patterns (Table 1; Fig. 1) for the two tissues. At lower concentrations of eserine, up to 5.7 x 10^{-6}M, the inhibition of the enzyme activity in muscle was higher than in brain. However, at higher concentrations (6.4 x 10^{-6}M onwards) the inhibition pattern became similar. The percent inhibition of muscle AchE showed hyperbolic and brain AchE showed a biphasic pattern. There was a sudden increase in the inhibition of brain AchE activity from 5.12x10^{-6}M eserine. In both cases almost complete inhibition was obtained at higher concentrations of the inhibitor.

The apparent Km (Michaelis constant) and Vmax for acetylthiocholine iodide were also different for brain and muscle AchE (Fig. 2; Table 2). Km was 7.27x10^{-5} and 8.9x10^{-5}M for brain and muscle AchE, respectively. Eserine showed competitive type of binding with acetylthiocholine iodide as

the substrate for both brain and muscle AchE (Fig. 2), - higher eserine doses increased the Km but not the Vmax.

Table 1. Effect of increasing eserine concentration on the specific activity (units/mg protein) x 10^3 of AchE **in vitro** from brain and muscle of **H. fossilis.** (Values are expressed as Mean ±S.D. The level of significance (p) at each concentration was calculated by comparison with the control value and using student 't' test).

Concentration of Eserine(M)	AchE Specific Activity	
	Brain	Muscle
0 (Control)	33.76 ± 0.09	49.03 ± 0.19
0.320×10^{-6}	-	47.92 ± 0.32
		p 0.05
1.28×10^{-6}	33.56 ± 0.48	40.42 ± 0.10
	(N.S.)	p 0.05
2.56×10^{-6}	32.89 ± 0.28	-
	p 0.05	
3.20×10^{-6}	32.45 ± 0.11	30.75 ± 0.39
	p 0.01	p 0.01
5.12×10^{-6}	31.00 ± 0.37	27.38 ± 0.12
	p 0.01	p 0.01
5.76×10^{-6}	22.77 ± 0.19	-
	p 0.01	
6.40×10^{-6}	11.66 ± 0.11	17.82 ± 0.57
	p 0.01	p 0.01
12.8×10^{-6}	4.40 ± 0.11	6.09 ± 0.10
	p 0.01	p 0.01
32.0×10^{-6}	0.77 ± 0.11	1.22 ± 0.13
	p 0.01	p 0.01
64.0×10^{-6}	0.77 ± 0.11	1.22 ± 0.13
	p 0.01	p 0.01

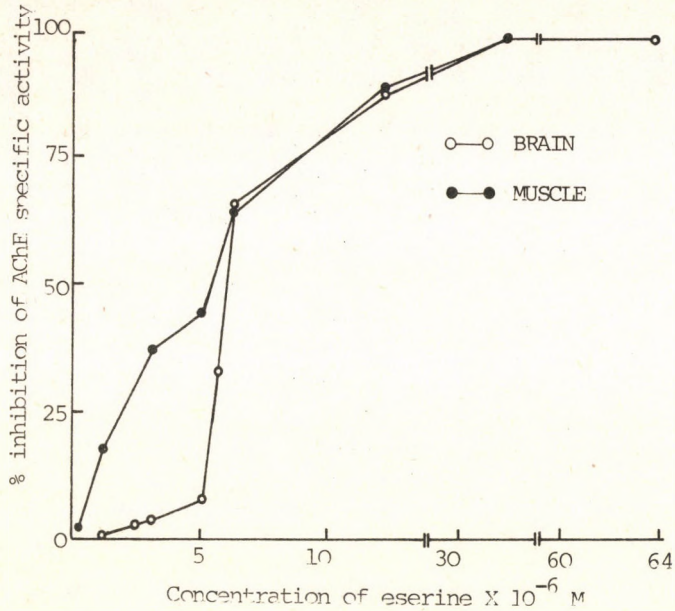


Fig. 1. Percent inhibition of specific activity of AchE in brain and muscle homogenates by different concentrations of eserine.

Specific staining on polyacrylamide gel indicated only one major tissue specific AchE band each in brain and muscle homogenates and some indistinct minor bands by both visual observation and densitometric scanning (Fig. 3).

Table 2. Effect of eserine on the apparent Km and Vmax of AchE in brain and muscle of **H. fossilis** using acetylthiocholine iodide as substrate.

	Tissue	Eserine Concentration x 10^{-6}M			
		0	3.2	5.1	6.4
Km x 10^{-5}M	Brain	7.27	-	24.24	32.00
	Muscle	8.90	13.60	-	25.89
Vmax x 10^{3} (units/mg protein)	Brain	44.40	-	44.40	44.40
	Muscle	58.80	58.80	-	58.80

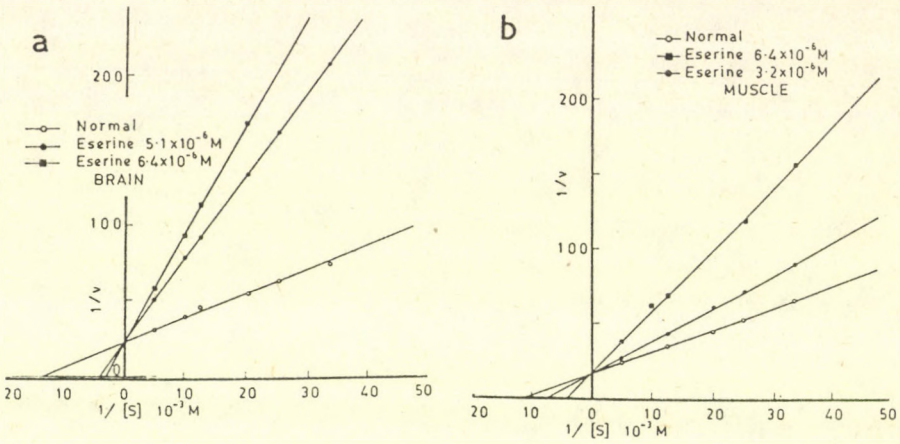


Fig. 2. Lineweaver - Burk plot for determination of Km of AchE for acetylthiocholine iodide, also demonstrating the nature of eserine binding in (a) brain and (b) muscle of **H. fossilis.**

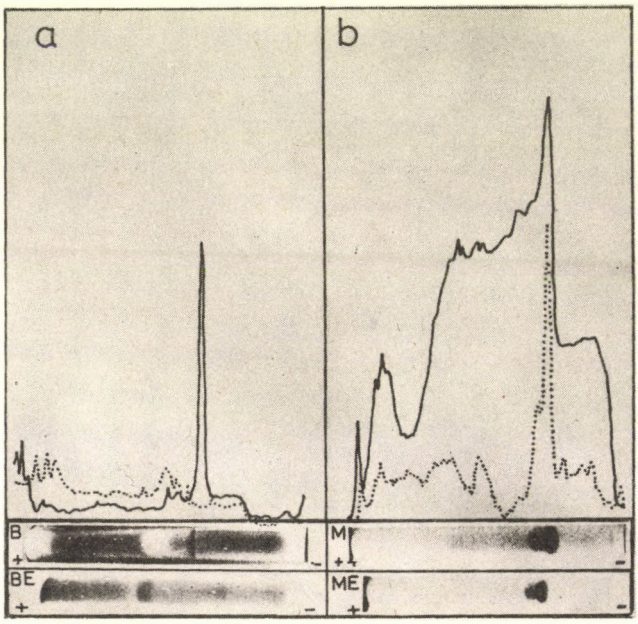


Fig. 3. Densitometric absorption of polyacrylamide gels specifically stained for AchE ac tivity with acetylthiocholine iodide (_____) and eserine + acetylthiocholine iodide (---) in (a) brain and (b) muscle homogenates of **H. fossilis.**

DISCUSSION

Tissue specific forms of AchE have been reported to exist in several animals. Three forms (18S, 12S and 5S) of AchE were reported in both brain and muscle tissues of gold fish, **Carassius auratus** (Guillon, Masoulie, 1976). However, 18S was the predominant form in the muscle and 12S in the brain. The activity levels of the three forms changed under different physiological states of the animal. In rat diaphragm muscle, 4 forms of AchE (16, 12.6,9 and 4S) were detected out of which the 16S form was found to be associated with neuro-muscular junction (Hall, 1973; Bernsohn et al., 1963). The monomeric 4S and functional 16S forms were predominant whereas the other two froms accounted for a very little activity (Hall, 1973). AchE of chick brain had also 4 molecular forms (17,11,6 and 4S) of which 11S was the dominant form (Scarsella et al., 1978). Different reports indicate that AchE exists in multiple molecular forms in different tissues out of which generally one remains as predominant tissue specific enzyme. Our result (Fig. 3) showed one major band of AchE each in brain and muscle besides some minor bands. The major bands showed different migration on the gel indicating that structurally they are different and represent their tissue specific form. The electrophoretic method used is not sensitive enough to detect all the low activity molecular forms. Therefore, it is difficult to say exactly how many molecular forms of AchE existed in the two tissues studied.

The specific activity, Km, Vmax and pattern of inhibition by eserine were different in brain and muscle AchE (Tables 1,2; Fig. 1,2). The results indicated that the physiological level, and binding affinity with the substrate and the inhibitor were significantly different in the two forms of AchE. Different isoenzyme forms have been known to vary in their activity and regulation. The response of different forms of AchE to the inhibitor has been shown to be different. Injection of some inhibitors such as parathion inhibited the activity of AchE differently in different tissues (Vijaylakshmi et al. 1977). This difference was correlated with the isoenzymic variations of the enzyme in different tissues. The hyperbolic inhibition pattern of muscle and biphasic inhibition pattern of brain AchE by eserine obtained in the present study also indicated functional difference of AchE in the two tissues. The biphasic pattern might be due to different catalytic units or different intra-molecular interactions of

brain AchE. Bon and Massoulie (1976) reported six different molecular forms of AchE in **Electricus** which were distinguished into two classes. One class (18.4, 14.2 and 9S) was highly asymmetric and the other class (11.8,7.7 and 5.3S) showed no such asymmetry. The asymmetric molecules had a rod like tail and it was suggested that these two groups might be functionally different. In another report (Gentinetta, Brodbock, 1976), 11S AchE which was usually the predominant form in brain was separated into two non-identical sub-units with different catalytic activities. Barnett and Rosenberry (1979), however, could not show any functional difference between the two sub-units. The present results support the former suggestion. Brain AchE might exist in two catalytically different forms having different binding affinity for eserine or the sub-units might be identical showing cooperativity in their binding with eserine.

The Km for acetylthiocholine iodide of brain and muscle AchE were found to be of lower order in comparison to the values already known for mammalian and **Electricus** AchE (Mohon, Brink, 1970; Giri et al., 1977; Moss, Fahrney, 1978) indicating higher catalytic efficiency of **H. fossilis** AchE. The lower level of AchE activity observed in the tissues might have been compensated by the higher catalytic efficiency of the enzyme for its physiological role. The Km values reported by different authors vary so much that it is not possible to accept any one as standard. These variations might be due to the physiological adaptations of different species to their respective environment. However, the binding of eserine was competitive in nature in all the AchE studied. The variations in specific activity, inhibition pattern by eserine, kinetic values and electrophoretically separable forms of the enzyme suggest physiological differentiation of AchE in brain and muscle of **Heteropneustes fossilis.**

Acknowledgement

The authors thank the Head of the Department of Zoology for providing the facilities. The work was partly financed by Council of Scientific and Industrial Research, India.

REFERENCES

Baldwin, J., Hochachka, P.W. (1970) Biochem. J. 116, 883-887

Baldwin, J. (1971) Comp. Biochem. Physiol. 40, 181-187

Barnett, P., Rosenberry, T.L. (1979) Biochim. Biophys. Acta 567, 154-160

Bernsohn, K.D., Barron, J., Hess, A.R., Hendrick, M.T. (1963) J. Neurochem. 10, 783-794

Bon, S., Huet, M., Lemonnier, M., Rieger, F., Massoulie, J. (1976) Eur. J. Biochem. 68, 523-530

Bon, S., Massoullie, J. (1976) FEBS Lett. 71, 273-278

Carson, S., Bon, S., Vigny, M., Massoulie, J., Fardean, M. (1979) FEBS Lett. 97, 348-352

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427

Ellman, G.L., Courtney, K.D., Andres, V., Fetherstone, R.M. (1961) Biochem. Pharmacol. 7, 88-95

Fluck, R.A. (1978) Develop. Growth and Differ. 20, 17-25

Gentinetta, R., Brodbock, U. (1976) Biochim. Biophys. Acta 438, 437-448

Giri, N., Hollinger, M.A., Schiedt, M.J. (1977) Biochem. Pharmacol. 26, 313-317

Gisieger, V., Vigny, M., Goutron, J., Rieger, F. (1978) J. Neurochem. 30, 501-516

Guillon, G., Massoulie, J. (1976) Biochimie 58, 465-471

Hall, Z.W. (1973) J. Neurobiol. 4, 343-361

Koelle, G.B. (1951) J. Pharmacol. Exp. Therap. 103, 153-171

Lappin, R.I., Rubin, L.L. (1985) Dev. Biol. 110, 269-274

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Markert, C.L. (1968) Ann. N.Y. Acad. Sci. 103, 915-929

Maynard, E.A. (1964) J. Exp. Zool. 157, 251-266

Michalek, H., Pintor, A., Sostuna, S., Bisso, G.M. (1984) Acta Biochim. Pol. 31, 149-153

Mohon, P.J., Brink, J.J. (1970) J. Neurochem. 17, 949-953

Moss, D., Fahrney, D. (1978) Biochem. Pharmacol. 27, 2693-2698

Moudgil, V.K., Kanungo, M.S. (1973) Biochem. Biophys. Acta 329, 211-220

Nachmanshon, D. (1959) Chemical and molecular basis of nerve activity, Acad. Press Inc., New York

Principato, G.B., Ambrosini, M.V., Menghini, A., Giovannini, E., Dellagata, M. (1978) Comp. Biochem. Physiol. 61, 147-151

Ramanujam, S.N., Ratha, B.K. (1981) Comp. Physiol. Ecol. 6, 201-205

Ramanujam, S.N., Ratha, B.K. (1983) Proc. Indian Natl. Sci. Acad (1983) 49, 93-100

Rider, C.C., Taylor, C.B. (1980) Isoenzymes, Chapman et Hall Pub.

Rieger, F., Bon, S., Massoulie, J., Cartaud, J., Picard, B., Benda, P. (1976) Eur. J. Biochem. 68, 513-521

Scarsella, G., Toschi, G., Chiappinelli, V.A., Giacobini (1978) Dev. Neurosci. 1, 133-141

Vigny, M., Koening, J., Rieger, F. (1976) J. Neurochem. 27, 1347-1353

Vigny, M., Bon, S., Massoulie, J., Gisilger, V. (1979) J. Neurochem. 33, 559-565

Vijayan, V.K., Brownson, R.H. (1975) J. Neurochem. 24, 105-110

Vijaylakshmi, S., Pavankumar, T., Muralimohan, P., Babu, S.K. (1977) Comp. Physiol. Ecol. 2, 58-61

Weinberg, C.B., Hall, Z.W. (1979) Dev. Biol. 68, 631-635

Whittaker, J.R. (1979) Biol. Bull. 157, 344-355

Acta Biochim. Biophys. Hung. 21/4/, pp. 391-395 /1986/

EFFECT OF GLYCEROL ON THE LOW-TEMPERATURE FLUORESCENCE SPECTRA OF GREEN ALGAE

(Short Communication)

P. Butko, L. Szalay

Department of Biophysics, József Attila University, Szeged, Hungary

(Received July 25, 1986)

Glycerol is a common cryoprotectant (Packer, Barnard, 1966). It is added to samples prior to low-temperature optical measurements in order to prevent crystallization (Rijgersberg, 1980). However, we found that upon addition of glycerol to a suspension of algae, the organization of the photosynthetic apparatus is markedly changed. Also the regulation of the excitation energy distribution between the two photosystems (PSs) is impaired.

The alga. Chlorella pyrenoidosa (strain Emerson) was grown as described previously (Butko, 1984).

Fluorescence measurements. The emission spectra of chlorophyll a were measured in a glass cell with a diameter of 3 mm at 77 K with a Perkin-Elmer MPF-44A spectrofluorimeter, at an excitation wavelength of 485 nm. The samples contained 52 per cent (v/v) glycerol.

State 1/State 2 transitions. The notions of State 1 and State 2 have been introduced (Bonaventura, Myers, 1969) to describe the energy distribution between PSII and PSI. State 1 is the state which develops under illumination with the light preferentially absorbed by PSI: PSI being overexcited and PSII lacking energy, a regulatory mechanism - trying to overcome the inequilibrium - will deliver more energy to PSII in this state than in State 2. In State 2 the situation is just the opposite.

The samples were adapted to State 1 and State 2 through illumination with 710 nm and 650 nm light, respectively (intensities ca. 300 $\mu W/cm^2$), for 15 min.

Abbreviation: PS, photosystem.

Akadémiai Kiadó, Budapest

The three characteristic bands in the low-temperature spectra of algae, denoted F686, F697 and F724, have been assigned to the antenna system of PSII and the light-harvesting complex, the reaction center of PSII, and the chlorophylls of PSI, respectively (Lavorel, Etienne, 1977; Breton, 1982). The fluorescence ratio F686/F724 is thus a convenient parameter reflecting the relative energy utilization in PSII and PSI: a high F686/F724 ratio indicates State 1, whereas a low F686/F724 ratio reflects State 2 (Murata, 1969).

We found that the extent of the State 1/State 2 transitions in the presence of glycerol is less than a half of that in its absence (Table 1).

Table 1.

Effect of glycerol on the extent of the state transitions

The extent of the state transitions was evaluated from the 77 K chlorophyll fluorescence spectra as a change in the PSII/PSI fluorescence ratio $([F686/F724]_{St1} - [F686/F724]_{St2})/[F686/F724]_{St1}$.

State	Glycerol	F686/F724	Extent of the transition
1	-	0.79	
2	-	0.59	0.25
1	+	0,97	
2	+	0.86	0.11

A similar observation was mentioned also by Saito et al. (1983). They hypothesized that the transitions are impaired due to osmotic effects. However, not only development of State 1 or State 2 (i.e. the energy distribution between PSII and PSI) is affected by glycerol: the ratios of the peak heights themselves change in the presence of glycerol.

The data of Fig. 1 show that: (i) the incubation with glycerol causes a progressive quenching of the PSII fluorescence and (ii) the fluorescence of the antenna moleculess (F686) is quenched faster than that of the PSII reaction center (F697). It suggests that glycerol directly affects the photosynthetic apparatus, apparently diminishing the size of the PSII antenna.

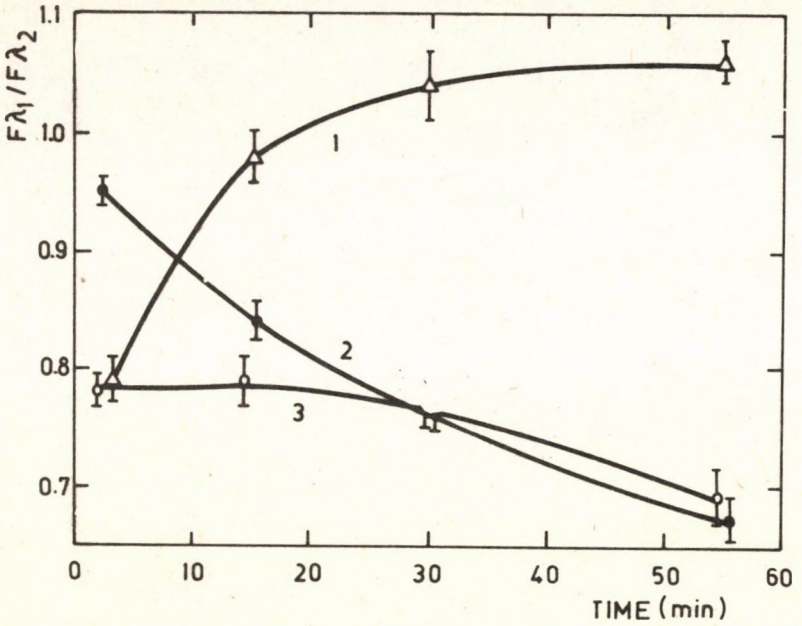


Fig. 1. Effect of incubation with glycerol (52%) on the low-temperature fluorescence spectra (the peak ratios) of Chlorella. Curve 1 (Δ), F697/F686; curve 2 (●), F686/F724; curve 3 (o), F697/F724. Every point represents the average of at least 5 measurements, standard error of the mean being indicated.

Cryoprotection. Samples with and without glycerol were frozen to 77 K and after melting, refrozen again (Fig. 2). A qualitative analysis show that glycerol really protects the photosynthetic apparatus against damages caused by freezing-melting-refreezing process: curve 2 in Fig. 2 has all the characteristics of a normal low-temperature spectrum of green algae (curve 1), although small defects in PSII are indicated by lower fluorescence yields below 700 nm. In contrast, curve 3 which was recorded with a sample refrozen in the absence of glycerol shows a complete disorganization of the photosynthetic apparatus: fluorescence of PSII disappeared and the long-wavelength maximum was shifted from 724 nm to 719 nm. Ice crystals may have totally disrupted the thylakoids, nearly all chlorophyll probably behaved like free pigment. It is interesting to note that the damage is a consequence of melting rather than that of freezing itself.

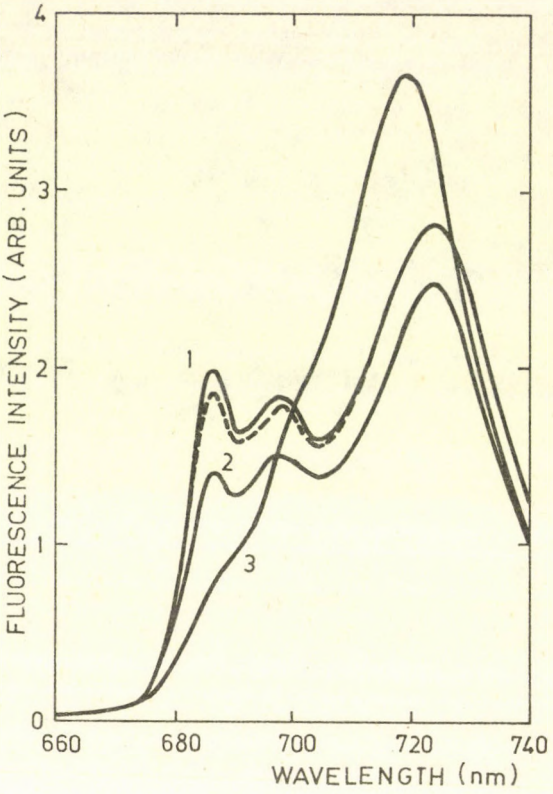


Fig. 2. Protective effect of glycerol against freezing damages to the photosynthetic apparatus. Curves 1, samples with (----) and without (_____) 52% (v/v) glycerol, the spectra being recorded after the first freezing to 77 K; curve 2, sample with glycerol after repeated freezing; curve 3, sample without glycerol after repeated freezing.

Viability of the cells. Cho and Govindjee (1970) have already observed that the viability of the rewarmed algae is poor. We experienced that a mere addition of 29 per cent (v/v) glycerol to the medium inhibits the culture growth (Table 2).

Table 2.

Effect of glycerol on the growth of the algal culture

Time (days)	Cell density (10^6 ml^{-1}) Control	+ glycerol (29%)
0	6	6
1	32	6

On the basis of these few experiments and equally few scattered literary data (Saito et al., 1983; den Haan, 1976; Barnett, 1978), we do not feel entitled to speculate about a possible mechanism of the effect of glycerol on the photosynthetic apparatus. Nevertheless, we certainly can give warning against a careless use of glycerol in the spectroscopy of photosynthesizing objects.

REFERENCES

Barnett, R.E. (1978) Cryobiology 15, 227-229

Bonaventura, C., Myers, J. (1969) Biochim. Biophys. Acta 189, 366-383

Breton, J. (1982) FEBS Lett. 147, 16-20

Butko, P. (1984) Photobiochem. Photobiophys. 8, 63-72

Cho, F., Govindjee (1970) Biochim. Biophys. Acta 205, 371-378

den Haan, G.A. (1976) Thesis, Leiden

Lavorel, J., Etienne, A.L. (1977) in: Primary Processes of Photosynthesis, Barber, J. ed., Elsevier, Amsterdam, pp. 203-268

Murata, N. (1969) Biochim. Biophys. Acta 172, 242-251

Packer, L., Barnard, A.C. (1966) Biochim. Biophys. Acta 126, 443-448

Rijgersberg, C.P. (1980) Thesis, Leiden

Saito, K., Williams, W.P., Allen, J.F., Bennett, J. (1983) Biochim. Biophys. Acta 724, 94-103

BOOK REVIEWS

MULTIDOMAIN PROTEINS edited by L. Patthy and P. Friedrich, Proceedings of the UNESCO Workshop on Structure and Function of Proteins, Budapest, September 13-15, 1984, Akadémiai Kiadó (Publishing House of the Hungarian Academy of Sciences), Budapest, pp. 223, 26.00 $ (260 Ft)

Owing to the development of the automatization of the sequencing and synthesis of proteins and nucleic acids and to that of other sophisticated techniques the possibility of looking into the intimate details of the structure of biological macromolecules has been greatly enhanced during the last decade. By this means we have acquired several pieces of knowledge that have changed our previous ideas concerning the structure of these materials in many respects. It has become clear that the information determining the amino-acid sequencing of quite a great number of recent proteins has got formed by gene fusion, gene duplication and similar evolutionary events.

In consequence of the linkage and multiplication of the ancient primitive genes, domains of different biological value have got formed in the structure of recent proteins. The function of some of these is already understood, but not of all.

The above questions are treated from diverse aspects in the 16 papers read at the symposium. Probably this is one of the outstanding merits of the volume. There has been drawn a very colourful, manysided picture to interpret the significance and evolutionary development of the domain structures by means of comparing the X-ray results with the theoretical considerations, the analyses of the evolutionary possibilities with the information contained in mRNA by the formation of the functional characteristics as well as by comparing the physical-chemical or structural basis of these.

The General Discussion at the end of the volume is about 19 pages indicating that the presentations were followed

by lively disputes to sum up the present day point of view and outline the most urgent tasks to be solved. It is a pity that the volume could only be published one and a half year after the papers had been delivered.

P. Elődi

DINAMICS OF BIOCHEMICAL SYSTEMS edited by S. Damjanovich, T. Keleti and L. Trón. Akadémiai Kiadó, The Publishing House of the Hungarian Academy of Sciences, Budapest, 1986, 562 pages (ISBN 963 05 4356 7)

The book appeared in the series Symposia Biologica Hungarica as Vol 30 and contains the lectures presented at the FEBS Advanced Course and the Round Table Discussion of the IUB Interest Group on Kinetics and Mechanisms of Enzymes and Metabolic Networks held at Debrecen (Hungary) on 18-24th August, 1985. The scientific material has been organized in five chapters:

1. Kinetics and mechanism of enzymes and metabolic networks (4 papers),
2. Dynamics of protein and nucleic acid structure (5 papers),
3. Dynamics of multienzyme complexes and metabolic pathways (8 papers),
4. Enzyme kinetics and regulation (4 papers),
5. Membrane dynamics (12 papers).

The first chapter is introduced by a theory of enzyme evolution that is followed by kinetic analysis of the glycolytic pathway and mitochondrial ion transport. The main topics of chapter 2. are fluctuation of proteins, allosteric enzymes and DNA conformation. In section 3. the questions of metabolite compartmentation and channeling, as well as

the interplay between metabolic sequences are discussed in detail. It is demonstrated by several examples that not only the components of multienzyme complexes but also so called soluble enzymes are bound together by protein-protein interactions and the formation of loose aggregates has profound influence on the catalytic and regulatory properties of the constituent enzymes. Chapter 4. deals with model discrimination and estimation of kinetic parameters. These considerations, although purely theoretical, have great value in the design of kinetic experiments. Chapter 5. forms the largest and most complex part of the volume, since the membranes themselves are complex structures and the dynamics of their lipid, protein and glycoprotein components governs such complicated processes as translocation, endocytosis, secretion and transmembrane signalling. The influence of the cytoskeleton, membrane potential, receptor agonists and drugs on the properties of membranes is described in this section. Special attention has been given to the studies of the Fc receptors and antibiotics' action.

The well-illustrated lectures have been extended by references and discussion material. At the end of the volume a list of participants and a short subject index is also provided.

Papers on methodology, experimental research, computer simulation and intriguing hypothesis form a colourful selection of the latest results obtained in the field of macromolecular dynamics. This book will serve as a valuable source of information for enzymologists, immunologists, transport physiologists, cell biologists and others working in the interdisciplinary fields of biochemistry and biophysics.

V. Dombrádi

PRINTED IN HUNGARY
Akadémiai Kiadó és Nyomda, Budapest

CAVINTON®

INJECTION, TABLETS

Chemical Works of Gedeon Richter Ltd.
Budapest — Hungary

Cavinton improves cerebral metabolism

- — Increases the oxigen-utilization of the brain tissue
- — Increases the anoxia-tolerance of the brain cells
- — Shifts to aerobic way the glucose metabolism
- — Inhibits phosphodiesterase activity
- — Stimulates adenylcyclase activity increasing cAMP concentration of the brain cells
- — Increases ATP concentration

Exported by Medimpex
Hungarian Trading Company
for Pharmaceutical Products

Cavinton improves microcirculation

- — Inhibits adenosine uptake of the RBC
- — Inhibits platelet aggregation
- — Decreases high blood viscosity
- — Increases RBC deformability
- — Promotes the O_2 transfer to the tissues
- — Stimulates glucose penetration through the blood-brain barrier

Cavinton increases cerebral blood flow

- — Increases selectively and intensively the CBF
- — No steal-effect
- — Inverse steal-effect
- — Does not cause bradycardia or hypotension
- — Increases vasodilatation induced by hypoxia

Medicina 30.5090 — VBKM Nyomda — 86081

Biological Chemistry
Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

This Journal was founded in 1877 as *Zeitschrift für Physiologische Chemie* by F. Hoppe-Seyler and was continued after his death under the editorship of A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* [Volume 21 (1895) – Volume 365 (1984)].

Subscription price: 1986, Volume 367 (12 issues): DM 880,–

Volume 367 June 1986 Number 6

Walter de Gruyter · Berlin · New York

Biological Chemistry
Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

This Journal was founded in 1877 as *Zeitschrift für Physiologische Chemie* by F. Hoppe-Seyler and was continued after his death under the editorship of A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* [Volume 21 (1895) – Volume 365 (1984)].

Subscription price: 1986, Volume 367 (12 issues): DM 880.–

Volume 367 | August 1986 | Number 8

The issue contains further papers from the laboratories of
L. Jaenicke, H. Grisebach, H. Fritz, P. Dimroth and W. Stoffel

Walter de Gruyter · Berlin · New York

Biological Chemistry
Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

This Journal was founded in 1877 as *Zeitschrift für Physiologische Chemie* by F. Hoppe-Seyler and was continued after his death under the editorship of A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* [Volume 21 (1895) – Volume 365 (1984)].

Subscription price: 1986, Volume 367 (12 issues): DM 880,–

Volume 367 | October 1986 | Number 10

Walter de Gruyter · Berlin · New York

Biological Chemistry Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

This Journal was founded in 1877 as *Zeitschrift für Physiologische Chemie* by F. Hoppe-Seyler and was continued after his death under the editorship of A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* [Volume 21 (1895) – Volume 365 (1984)].

Subscription price: 1986, Volume 367 (12 issues): DM 880,–

Volume 367 | December 1986 | Number 12

Walter de Gruyter · Berlin · New York

A desirable plan for the organization of a paper is the following: Summary, Introduction, Materials and methods, Results, Discussion, References.

A clear and informative title is very important. Provide a short title (not to exceed 50 characters and spaces) to be used as a running head. Every paper must begin with a **Summary** (up to 200 words) presenting the important and pertinent facts described in the paper.

The **Introduction** should state the purpose of the investigation, but should not include an extensive review of the literature. The description of the **Materials and methods** should be brief, but adequate for repetition of the work. Refer to previously published procedures employed in the work. It is strongly recommended that author(s) should draw attention to any particular chemical or biological *hazards* that may occur in carrying out experiments described in the paper. Relevant safety precautions should be suggested. The **Results** may be presented in tables or figures. The **Discussion** should be concise and deal with the interpretation of the results. In some cases combining **Results and discussion** in a single section may give a clearer presentation.

References to the literature in the text should be by numbers in parentheses. In the reference list the items should be arranged in order of these serial numbers. For citation note the following examples:

1. Drust, D. S. and Martin, T. F. J. (1985) Biochem. Biophys. Res. Commun. 128, 531–537
2. Hoyer, S. (1980) in Biochemistry of Dementia (Roberts, P. J. ed.) pp. 252–257, J. Wiley and Sons, New York

Abbreviations and symbols defined in the IUPAC-IUB Document No. 1 (Arch. Biochem, Biophys. *115*, 1–12, 1966) may be used without definition, but others are to be avoided. When necessary, *abbreviations must be defined in a footnote and typed single-spaced on a separate page*, rather than in the text. Abbreviations *must not* be used in the Summary. Enzyme names should not be abbreviated except when the substrate has an accepted abbreviation (e.g. ATPase). Use of Enzyme Commission (EC) code number is required when available. Styling of isotopes should follow the recommendations of the IUB Commission of Editors. The symbol of the isotope should be placed in brackets attached directly to the front of the name, e.g. [^{32}P] AMP. Isotope number should only be used as a (superior) prefix to the atomic symbol, not to an abbreviation.

Typing of manuscript

The accepted articles are reproduced directly from the submitted manuscript as camera ready copy, thus *no changes can be made in the manuscript after acceptance*. Therefore black silk or carbon typewriter ribbon should be used. For obvious reasons proofs are not required and cannot be supplied. Special care should be taken to ensure a sharp, clean impression of letters throughout the whole manuscript. It is strongly recommended to use an *electric typewriter*. Erasures or other corrections should be avoided. Spelling, word division and punctuation must be carefully controlled.

The title of the paper should be typed in capital letters near the top of the first page, with the name(s) an affiliation(s) of the author(s) just typed below. Manuscripts should be double-spaced; methods, references, abbreviations, footnotes and figure legends should be *single-spaced*. Typing area of the page must be as close as possible to 16 × 24 cm.

Each page should be numbered at the bottom in light blue pencil marks. On a separate sheet indicate a running title, not more than 6 key-words for subject indexing and the author to whom reprints are to be sent, including a telephone number if possible.

Illustrations and tables. Do not attempt to insert figures, figure legends or tables into the text. This will be done by the Publisher. Original drawings should be clearly labelled in black ink in a manner suitable for *direct reproduction*. Typewritten lettering is not acceptable. Figures will be reduced to fit within the area of the page. All letters, numbers and symbols should be drawn to be at least 2.5 mm high after reduction. Glossy photographs or drawings are acceptable provided they are sharp, clear prints with an even black density. Illustrations in colour cannot be accepted. Mathematical and chemical symbols that cannot be typed should be drawn carefully in black. Tables and illustrations should be submitted on separate sheets.

One hundred reprints will be supplied free of charge.

CONTENTS

Index: 26.030