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ISOLATION OF HIGH MOLECULAR WEIGHT PLANT NUCLEAR DNA SUITABLE FOR USE IN RECOMBINANT DNA TECHNOLOGY

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(Received October 31, 1986)

SUMMARY

Nuclei isolated from leaf cells of broad bean (Vicia faba L) by a newly developed method based on the use of citric acid in the isolation medium and floatation on a Percoll cushion yielded high molecular weight plant nuclear DNA which was suitable for (i) analysis by restriction endonucleases, (ii) molecular cloning and (iii) genomic blot hybridization. Starting from nuclear preparations obtained by this method, U2 small nuclear RNA-specific DNA sequences were detected in Vicia faba L. This is the first report on the demonstration of small nuclear RNA-specific DNA sequences in plant material.

INTRODUCTION

Over the past few years great effort has been made to understand the nuclear gene structure and expression in higher plants. Such studies require an increasing use of recombinant DNA technology. However, there are a number of methodological difficulties in the isolation of pure and undegraded plant nuclear DNA, an essential prerequisite for the construction of plant genomic libraries or for analyses of specific sequences by Southern blotting. Because of the necessity of using tough cell wall disruption techniques and of the ubiquitous presence of contaminating nuclease activity, plant DNA

Abbreviations: kbp, kilobase pairs; Mes, 4-morpholine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (150 mM NaCl/15 mM citric acid)

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tends to be degraded during the purification procedure. In addition, it is extremely difficult to remove the hardly detectable polysaccharide-like and the brown tannin contaminants that can easily interfere with the restriction enzyme activities and may alter the results of the particularly sensitive DNA reassociation experiments (Murray and Thompson, 1980). Therefore, purified nuclei seem to be the only suitable starting material for the isolation of high molecular weight pure plant nuclear DNA.

Several procedures have been described for the purification of plant nuclei. Although these methods are cumbersome and time consuming, generally they do not yield sufficiently pure and intact nuclei (Kiss et al., 1985).

We have developed a simple and rapid procedure for the isolation of plant nuclei. It is based on the use of citric acid in the isolation medium (Taylor et al., 1975). It works with a wide variety of green plants and cell suspension cultures. Subsequent isolation of high molecular weight DNA (a minor modification of the method by Blin and Stafford, 1976), gives rise to DNA preparations in which nuclease activity is practically eliminated and polysaccharides seem to be absent.

MATERIALS AND METHODS

Chemicals

Percoll was purchased from Pharmacia (Uppsala), cellulose "Onozuka" R-10 and Macerozyme R-10 from Kinki Yakult (Nischinomiya), SDS, EDTA, 2-mercaptoethanol, proteinase K, ribonuclease T_1 from Serva (Heidelberg), ribonuclease A from Sigma (St.Louis). All other chemicals were of analytical grade as obtained from Reanal (Budapest).

Isolation of nuclei

Plants were grown in a greenhouse at 25°C and 16 hours light period. Fully expanded leaves of 3-week-old broad bean (Vicia faba L) plants were used. All isolation steps were carried out at 4°C. The harvested leaves (about 100 g) were homogenized with an Ultra Turrax homogenizer at full speed for 4x15 s in 600 ml of isolation medium 15% (w/v) citric acid and 0.25% Triton X-100). The homogenate was sequentially filtered through four layers of cheese-cloth and two nylon meshes of 67 and 20 µm pore size (Züricher Beuteltuchfabrik AG).

The filtrate was centrifuged at 1000 x g for 10 min, and the pellet was washed with 100 ml of isolation medium and 50 ml of washing medium (25 mM citric acid, 250 mM sucrose and 0.7% (v/v) Triton X-100). After this step the supernatant was removed carefully and the pellet was suspended in 20 ml of Percoll adjusted to pH 6.5-6.8 by a few drops of isolation medium, and centrifuged at 4000 x g for 5 min in a swinging bucket rotor. The floating nuclear layer was taken off and washed twice with 50 ml of washing medium each time.

This procedure worked well with cell suspension cultures as well, except that prior to the isolation of nuclei a partial enzymatic digestion step had to be included. Lycopersicon peruvianum Mill. cell suspension cultures were maintained according to Nover et al. (1982) and used 3-4 days after passage. Cells (150 g wet weight) were collected on a nylon filter, rinsed with plasmolysis buffer (0.7 M mannitol/5 mM EDTA/10 mM Mes, pH 5.8) and resuspended in 300 ml of plasmolysis buffer supplemented with 0.1% cellulase and 0.05% Macerozyme. The suspension was incubated at 25°C for 3 h. The cells were washed with ice-cold isolation medium before homogenization.

Isolation of DNA

The nuclear pellet (2-4 x 10^8 nuclei) was lyzed at room temperature in 40 ml of lysis buffer (20 mM EDTA/10 mM NaCl/ 0.5% SDS/50 µg/ml proteinase K/20 mM Tris-HCl, pH 8). The highly viscous solution was incubated at 50°C for 4 h or at 37°C overnight, and extracted twice with an equal volume of distilled phenol saturated with 0.1 M Tris-HCl, pH 8.0/0.2% 2-mercaptoethanol. Centrifugations were performed in swinging bucket rotors for 10 min at 800 x g.

The aqueous phase was dialyzed against 3 liters of 50 mM Tris-HCl, pH 8.0/10 mM EDTA/10 mM NaCl, with several changes. The sample was removed from the dialysis bag, and deoxyribonuclease-free ribonuclease A and ribonuclease T_1 were added to a final concentration of 10 µg/ml and 5 U/ml, respectively. The ribonuclease digestion was performed at 37°C for 4 h.

The remaining protein contamination was removed by proteinase digestion. SDS and proteinase K were added to a final concentration of 0.5% and 50 µg/ml, respectively. The sample was incubated and extracted twice with phenol as described above. The volume of the aqueous phase was reduced by consecutive extractions with 2-butanol. Subsequently, the sample was dialyzed against 3 liters of 10 mM Tris-HCl, pH 8.0/1 mM EDTA, with several changes.

Gel electrophoresis

Horizontal agarose gels were run overnight at low voltage (0.5 1-V/cm). The electrophoresis buffer was 40 mM Tris/20 mM sodium acetate/2 mM EDTA, adjusted to pH 8.2 with glacial acetic acid. After electrophoresis the gels were stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light.

Restriction endonuclease and DNA ligase analyses

Restriction nucleases and T_4 DNA ligase were purchased from Boehringer (Mannheim) and used according to the manufacturer's recommendations.

Analysis of U2 RNA-specific sequences by genomic blot hybridization

Broad bean nuclear DNA (8 µg) was digested by restriction endonucleases and separated according to size by electrophoresis through 0.8% agarose gel. Transfer of DNA from agarose gel to nitrocellulose paper was performed according to Southern (1975). Purification and 3' end-labeling of broad bean U2 small nuclear RNA has been described earlier by Kiss et al. (1985). The genomic blot was hybridized with 3' end-labeled U2 small nuclear RNA at 42°C for 16 hours in a buffer containing 50% (v/v) formamide/50 mM K_2HPO_4/3 x SSC/5 x Denhardt's reagent (1 x Denhardt's reagent is 0.02% (w/v) bovine serum albumin) 0.02% (w/v) polyvinylpyrrolidone/0.02% (w/v) Ficoll).

RESULTS AND DISCUSSION

Our method for the isolation of nuclei (see Materials and Methods) yielded intact nuclei from broad bean, cucumber, tobacco and tomato leaves, as well as from Lycopersicon peruvianum and tobacco cell suspension cultures (not shown). Subsequent DNA purification resulted in high molecular weight plant nuclear DNA exhibiting A_{260}/nm A_{280}/nm ratios of 1.7-1.8. With a care in handling, plant DNA preparations migrated more slowly than intact lambda DNA, indicating a length of the order of 80-100 kbp (Fig. 1). The usual yield of nuclear DNA was about 1-3 mg/100 g plant material. These characteristics compare well with those reported in earlier (Sung and Slighton, 1981) and recent (Watson and Thompson, 1986) protocols, in both of which ultracentrifugation in CsCl gradients of the DNA is included.

Fig 2 shows that the high molecular weight plant nuclear DNA obtained by our method (lane E) contained neither endonuclease activity nor contaminants that could inhibit restriction enzyme activity, because, if codigested with lambda DNA, by using the appropriate amount of Hind III, the restriction pattern of lambda DNA (lane A) was not altered (lane B).

Apparently, our plant DNA is also suitable for molecular cloning because it contained neither exonuclease activity nor contaminants that could inhibit the activity of T4 DNA ligase. This is shown by the fact that an Eco RI digest of broad bean nuclear DNA (lane C) could be ligated to give rise to high

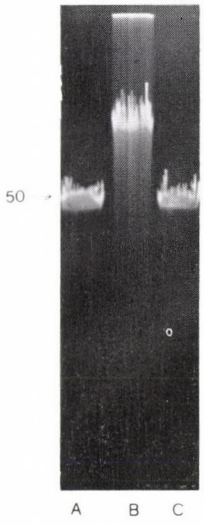

Fig. 1

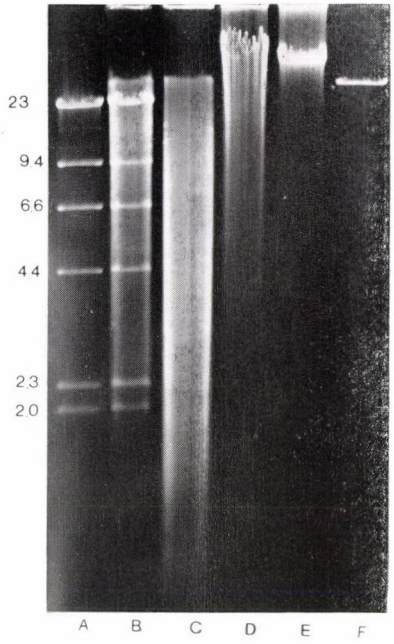

Fig. 2

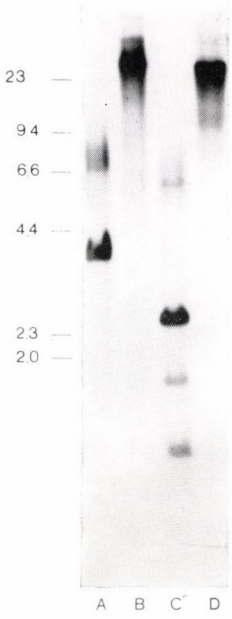

Fig. 3

Fig. 1. Agarose gel (0.3 %) electrophoretic pattern of broad bean nuclear DNA. Electrophoresis was carried out as described in Materials and Methods. As a molecular weight marker, 0.1 μg intact lambda DNA (lanes A and C) was run beside 0.2 μg broad bean nuclear DNA (lane B). The molecular size is given in kbp.

Fig. 2. Agarose gel (0.8 %) electrophoretic pattern of broad bean DNA extracted from purified nuclei, then digested with restriction endonucleases and subsequently treated with DNA ligase. Lane A, 0.3 μg of lambda DNA digested with 0.3 unit of Hind III for 1 h; lane B, 0.3 μg of lambda DNA and 1 μg of broad bean nuclear DNA digested with 1.5 units of Hind III for 1 h; lane C, 3 μg of broad bean nuclear DNA digested with Eco RI; lane D, 3 μg of broad bean nuclear DNA digested with Eco RI and ligated with T4 DNA ligase; lane E, 0.5 μg of untreated broad bean nuclear DNA; lane F, 0.05 μg of intact lambda DNA. The molecular sizes of the lambda Hind III fragments are given in kbp.

Fig. 3. Analysis of U2 small nuclear RNA-specific broad bean DNA sequences by genomic blot hybridization. Broad bean nuclear DNA (8 μg) was digested with Eco RI (lane A), or Hind III (lane B), or Bam HI (lane C), or Bgl II (lane D), fractionated by gel electrophoresis, blotted onto nitrocellulose filter and hybridized with labelled broad bean U2 small nuclear RNA as described in Materials and Methods. The molecular sizes of the lambda Hind III fragments are given in kbp.

molecular weight DNA (lane D) whose distance of migration compared well with that of untreated broad bean DNA (lane E). Finally, the nuclear DNA isolated from broad bean nuclei prepared by our method as described in this paper served as a reliable material for genomic blot hybridization, as shown in Fig. 3. In this experiment 3' end-labeled U2 small nuclear RNA from broad bean (Kiss et al., 1985) was used as a probe.

This is the first report on the detection of small nuclear RNA-specific DNA sequences in plants. Genomic libraries to be constructed from plants using nuclear DNA prepared by the method described in this paper will serve to detect and characterize genes and pseudogenes of plant small nuclear RNAs.

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Thank are due to Dr. L. Nover (Institut für Biochemie der Pflanzen, AdW, Halle) for supplying L. peruvianum callus tissues, to Mr. B. Dusha for preparing the photographs and to Mrs. Maria Doktor for typing the manuscript.

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THE EFFECT OF THE cAMP-DEPENDENT PROTEIN KINASE ON PROTEIN PHOSPHORYLATION AND PHOSPHATIDYLINOSITOL 4-PHOSPHATE FORMATION IN A HEPATOCYTE MEMBRANE PREPARATION

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(Received November 26, 1986)

SUMMARY

Plasma membrane preparation obtained from isolated mouse hepatocytes was phosphorylated by exogenous cyclic AMP dependent protein kinase in the presence of ^{32}P-ATP. The phosphorylated proteins were analysed by SDS-polyacrylamide gel-electrophoresis of the membrane and the phosphorylated lipids were analysed by thin layer chromatography of the separated lipid fraction. The protein kinase stimulated the ^{32}P incorporation into phosphatidylinositol 4-phosphate and it catalyzed the phosphorylation of several proteins. The main phosphoprotein bands were found at 51 kDa, 49 kDa, 46 kDa and 34 kDa. A part of the 51 kDa phosphoprotein accompanied the lipids in the course of the separation of the lipid fraction.

INTRODUCTION

The metabolism of the phosphorylated derivatives of phosphatidylinositol plays an important role in the regulatory mechanism of extracellular signals acting via the Ca^{2+} messenger system. A large group of such signals (hormones, neurotransmitters etc.) exerts its biological effects on cells by activating a phosphodiesterase which specifically breaks down

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Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate

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inositol-phospholipids in the plasma membrane to produce inositol trisphosphate (an intracellular messenger mobilizing calcium from internal stores) and diacylglycerol (an intracellular messenger activating protein kinase C) (reviewed in 1-4).

On the other hand, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate have been found to be powerful activators of a Ca^{2+}-ATP-ase (5, 6) which decreases the cytosolic calcium concentration.

The calcium messenger system and the cAMP messenger system are interrelated at several sites (reviewed in 7). We have reported that the cAMP-dependent protein kinase stimulates the phosphorylation of phosphatidylinositol to phosphatidylinositol 4-phosphate in the isolated plasma membranes of human lymphocytes (8) and thrombocytes (9), pig neutrophil granulocytes (10) and in a sarcoplasmic reticulum preparation from rabbit heart (11). However, the physiological significance of this effect of the cAMP-dependent protein kinase has not been clarified. In thrombocytes and neutrophil granulocytes cAMP and calcium represent antagonistic regulatory systems (7). In the heart cAMP potentiates the calcium signal but at the same time an important part of this effect is the stimulation of the temporary decrease of the cytosolic calcium concentration at the end of each contraction (7). We continued our investigations on hepatocytes because in the liver calcium and cAMP represent parallel regulatory systems acting in the same direction.

This paper shows that the cAMP-dependent protein kinase stimulated the phosphorylation of phosphatidylinositol in the isolated membrane of mouse hepatocytes. The phosphoproteins of the membrane were analysed as well.

MATERIALS AND METHODS

Preparation of isolated hepatocytes

Hepatocytes were isolated from CFLP mice (25-30 g body weight) by the collagenase perfusion method (12) as detailed previously(13). The viability of the cells controlled by the trypan blue exclusion test was about 90%.

Preparation of membranes

Plasma membrane and the endoplasmic reticulum were prepared by the slightly modified method of (14). Briefly, hepatocytes from two livers were suspended in 10 ml of ice cold 1 mM $NaHCO_3$ (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride and the cells were disrupted by 20-25 strokes of a Teflon-on-glass potter. The homogenate was mixed with 5.5 volume of 70.7% sucrose solution, 10 ml of aliquots were put in the tubes of an SW 27 rotor (Beckman) and 48.2% sucrose solution (15 ml) and then 42.5% sucrose solution (10 ml) were layered on the top of each aliquot. After centrifugation at 100 000 x g for 90 minutes the light gray layer formed at the 42.5-48.2% sucrose interface was removed, sedimented at 16 000 x g for 10 minutes, washed and suspended in 1 mM $NaHCO_3$ to a final concentration of 2-3 mg membrane protein per ml. This fraction was referred to as plasma membrane preparation.

The layer formed at the 48.2-60% sucrose interface was also washed and suspended to a final concentration of about 10 mg per ml protein and this fraction was referred to as endoplasmic reticulum preparation. The activity of 5'-nucleotidase (15) and the activity of glucose-6-phosphatase (16) were measured in the two types of membrane preparation and in the crude homogenate. 25-30 times higher 5'-nucleotidase specific activity and 5-10 times higher glucose 6-phosphatase specific activity were found in the plasma membrane preparation than in the crude homogenate. In the endoplasmic reticulum preparation these ratios were 5-8 and 15-20, respectively.

Preparation of the dissociated catalytic subunit of cAMP-dependent protein kinase

The catalytic subunit was prepared from rabbit skeletal muscle. 40 g of muscle was homogenized in a medium containing 0.04 M KCl/0.02 M $MgCl_2$/0.01 M Tris-HCl buffer (pH 7.5) and the protease inhibitor phenylmethylsulfonyl fluoride (2 mM). The crude extract was prepared and the DEAE-cellulose chromatography was carried out in the presence of cAMP as described in (17). Fractions containing the dissociated catalytic subunit were pooled, dialyzed against 5 mM Tris-HCl buffer (pH 7.5) and applied onto a phosphocellulose column (0.7 x 3.0 cm). Elution was performed by a NaCl concentration gradient in 10 mM Tris-HCl buffer (pH 7.5). The catalytic subunit was eluted at about 0.2 M NaCl. The specific activity of the preparation obtained was 50-120 nmol phosphate transferred x min^{-1}/mg protein when it was measured with H2B histone as a substrate.

The heat stable inhibitor protein of the catalytic subunit was prepared according to (18).

Phosphorylation of the membrane

Incubation media for the phosphorylation contained 8 mM potassium phosphate (pH 7.5), 15 mM $MgCl_2$, 12.5 μM ^{32}P-ATP (spec radioactivity 74 MBq/μmol), 150-300 μg membrane protein and 15-20 μg protein of the catalytic subunit preparation in a final volume of 0.4 ml. The incubation was carried out at 37°C for 5 min and it was stopped by trichloroacetic acid (15% final concentration). The precipitate was washed in trichloroacetic acid + ATP + P_i containing media, the membrane pellet

was dissolved in 5 ml of chloroform-methanol-HCl (100:100:0.6) solution, and a 1 ml aliquot was taken to measure the total ^{32}P-labelling. The remaining solution was mixed with 1 ml of 1 N HCl. The organic phase containing lipids was separated and the ^{32}P radioactivity was measured as in (7).

The phosphorylation of the membrane was carried out within 20 hours after membrane preparation. For this time the membrane was stored at -18°C.

The high performance thin layer chromatography of the lipid fraction was carried out as in (10).

The analysis of the phosphorylated membrane proteins by SDS-polyacrylamide gel-electrophoresis was performed on 12.5-15% polyacrylamide gels according to the method of (19). The protein kit of Pharmacia LMW was used for calibration.

RESULTS

Plasma membrane preparations obtained from isolated mouse hepatocytes were incubated in the presence of ^{32}P-ATP for 5 min. The incorporation of ^{32}P was measured in the total membrane and in the separated lipid fraction of the membrane. The ability of the membrane to incorporate ^{32}P into the lipid fraction varied significantly from preparation to preparation. In several preparations the ^{32}P-labelling found in the lipid fraction accounted for more than the 80 per cent of the total ^{32}P activity of the membrane, while in other preparations the ^{32}P-labelling of the lipid fraction was small. Table 1 demonstrates results obtained with membrane preparations belonging to the two different groups.

The dissociated catalytic subunit of the cAMP-dependent protein kinase increased the ^{32}P-incorporation into proteins in all cases. In preparations where the ^{32}P-labelling of the lipid fraction was responsible for a significant part of total ^{32}P-incorporation in the absence of the catalytic subunit, the addition of this enzyme strongly stimulated the lipid phosphorylation, too. Nevertheless, a slight increase of ^{32}P-labelling of the lipid fraction on the effect of the catalytic subunit was found practically in each preparation. The phosphorylated proteins were analysed by SDS polyacrylamide gel-electrophoresis. Fig. 1. shows a typical phosphoprotein pattern obtained in the presence of the catalytic subunit. Besides several minor bands a few major bands were observed at 51 kDa, 49 kDa, 46-1

Table 1. The effect of the cAMP-dependent protein kinase on the phosphorylation of the plasma membrane preparation obtained from isolated mouse hepatocytes

Preparations	Addition	^{32}P-incorporation (cpm per incubation mixture) total membrane	lipid fraction
preparation a	none	14 200	13 000
	catalytic subunit	96 000	41 400
preparation b	none	27 200	7 000
	catalytic subunit	44 000	9 000

Representative data of 12 experiments. 5 preparations showed results similar to preparation a and 7 showed results similar to preparation b.

kDa and at 34 kDa. A major band was also found in the range of the high molecular weight proteins. The membrane preparation did not contain endogenous cAMP-dependent protein kinase holoenzyme, since the addition of cAMP to the incubation mixture did not increase the ^{32}P-incorporation into the membrane.

Since the plasma membrane preparation was strongly contaminated by endoplasmic reticulum the phosphorylation pattern of an endoplasmic reticulum preparation (which was also contaminated by plasma membrane) was investigated as well (Fig.2). Similar phosphoprotein patterns were found, though the labelling of the 51 kDa protein and the 34 kDa protein seemed to be more intensive in the plasma membrane, while a 44 kDa phosphoprotein seemed to be more intensive in the endoplsmic reticulum preparation. The phosphorylation of the major bands was inhibited when the heat stable inhibitor protein of the cAMP-dependent protein kinase was also present in the incubation mixture (Fig. 3). The stimulated ^{32}P incorporation into the lipid fraction was also prevented by the heat stable protein (data not shown).

The analysis of the ^{32}P-labelled lipids was carried out in a high performance thin layer chromatographic system (Fig. 4). The identification of the ^{32}P-labelled lipids was based on the positions of unlabelled reference compounds run in the same system. The bulk of the ^{32}P-labelling was found in phosphatidylinositol 4-phosphate, though labelled phosphatidylinositol 4,5-bisphosphate was also detected. An unknown ^{32}P-labelled fraction just before phosphatidylinositol 4,5-bisphosphate was also observed. Though this fraction was not identified by a reference compound it might be a derivative of phosphatidylinositol 4-phosphate.

On the effect of the cyclic AMP-dependent protein kinase the ^{32}P incorporation into phosphatidylinositol 4-phosphate was increased significantly. This result shows that in the membrane of mouse hepatocytes the cAMP-dependent protein kinase stimulated the phosphorylation of phosphatidylinositol to phosphatidylinositol 4-phosphate. The stimulation of the formation of phosphatidylinositol 4,5-bisphosphate could not be detected, while the increased incorporation of ^{32}P into the small lipid fraction in the position just before phosphatidylinositol 4,5-bisphosphate was shown. The ability of phosphatidyl 4-phosphate formation to be stimulated by the cAMP-dependent protein kinase was a very sensitive property of the membrane and it was never observed if the membranes were stored for more than 24 hours after preparation.

The increased ^{32}P-labelling of a material remaining at the starting point in the course of thin layer chromatography of the lipid fraction was also observed. The increased ^{32}P-incorporation into this material on the effect of the catalytic subunit was demonstrated even in those membrane preparations where the ^{32}P-labelling of the lipid fraction was negligible. In order to clarify the nature of this material the following experiment was carried out. The phosphorylated membrane was dissolved in acidic chloroform methanol and the lipid fraction was separated from proteins by the usual method. The lipid fraction was dried and then it was prepared for SDS polyacrylamide gel-electro-

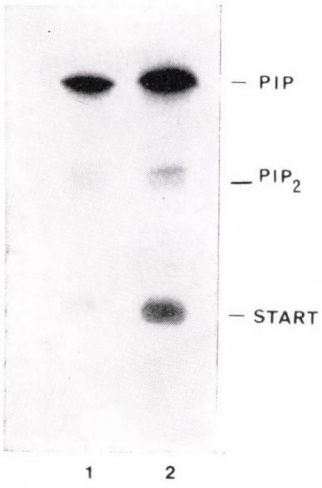


Fig. 4

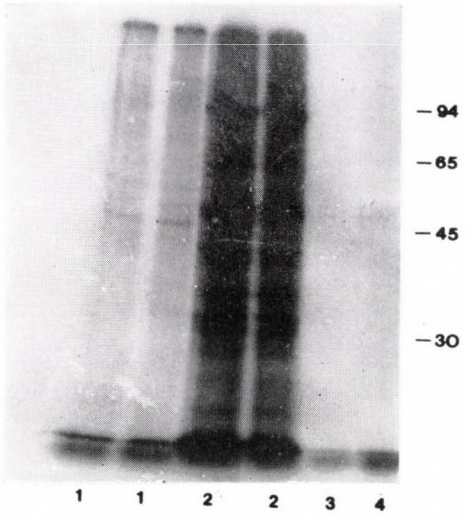


Fig. 5

Fig. 1. ^{32}P-labelled proteins of a hepatocyte membrane preparation phosphorylated by exogenous cAMP-dependent protein kinase. The phosphoprotein pattern obtained by the SDS-polyacrylamide gel-electrophoresis of the membrane was visualized by autoradiography. The molecular sizes (kDa) of the reference proteins are indicated by numbers.

1: autophosphorylation of the membrane; 2: phosphorylation in the presence of the catalytic subunit; 3: autophosphorylation of the membrane in the presence of cAMP (10^{-6}M); 4: autophosphorylation of the catalytic subunit preparation

Fig. 2. The phosphoprotein patterns of a plasma membrane preparation and of an endoplasmic reticulum preparation.

1: autophosphorylation of the plasma membrane preparation; 2: plasma membrane preparation phosphorylated by the catalytic subunit; 3: autophosphorylation of the endoplasmic reticulum preparation; 4: endoplasmic reticulum preparation phosphorylated by the catalytic subunit

Fig. 3. The effect of the heat-stable inhibitor protein of the catalytic subunit on the phosphorylation of the proteins of the plasma membrane preparation.

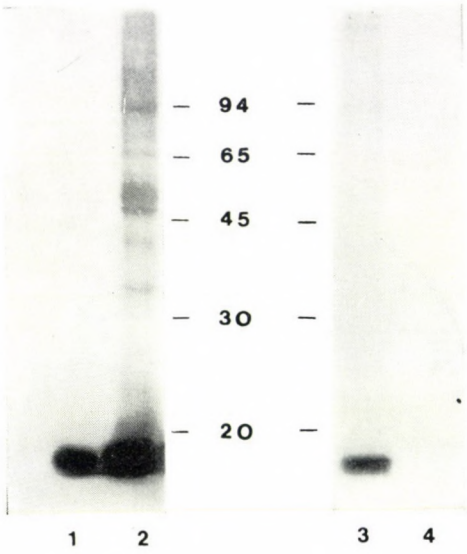
1: phosphorylation of the membrane in the presence of the catalytic subunit + heat-stable inhibitor protein; 2: phosphorylation in the presence of the catalytic subunit, 3: autophosphorylation of the membrane, 4: autophosphorylation of the membrane in the presence of the heat stable inhibitor protein

Fig. 4. ^{32}P-labelled membrane lipids separated by high performance thin layer chromatography. The ^{32}P-labelled lipids were visualized by autoradiography.

1: autophosphorylation of the plasma membrane preparation; 2: phosphorylation in the presence of the catalytic subunit

Fig. 5. SDS-polyacrylamide gel-electrophoresis of the separated lipid fraction.

1: autophosphorylation of the total membrane; 2: phosphorylation of the total membrane in the presence of the catalytic subunit; 3: ^{32}P-labelled components found in the lipid fraction of the autophysphorylated membrane; 4: ^{32}P-labelled components found in the lipid fraction of the membrane phpsphorylated in the presence of the catalytic subunit



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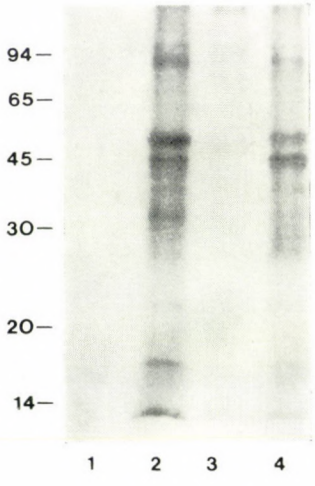

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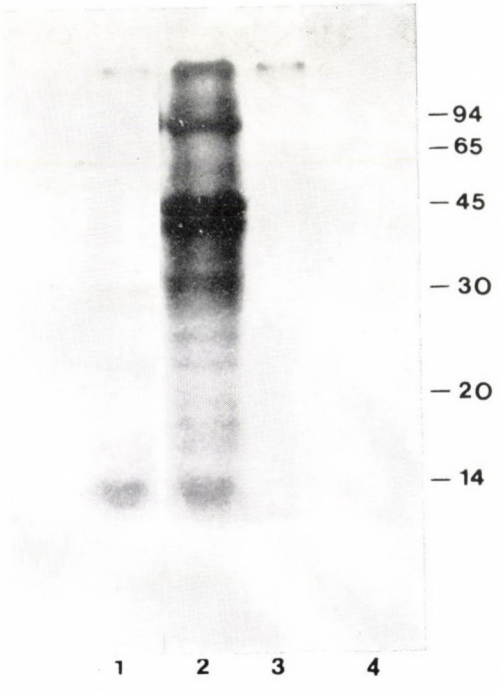

Fig. 3

phoresis. The gel-electrophoretic pattern of the ^{32}P-labelled components of the lipid fraction is shown on Fig. 5. Besides the ^{32}P-labelled lipids run just before the front, a ^{32}P-labelled band was also found in the position of the 51 kDa phosphoprotein which indicated that this major phosphoprotein of the membrane was at least partially soluble in the organic phase of the solvent mixture used. The appearence of the major phosphoprotein in the lipid fraction also explained the slight increase of ^{32}P-incorporation into the lipid fraction on the effect of the catalytic subunit in those membrane preparations where the lipid phosphorylation was negligible.

DISCUSSION

The hepatocyte membrane completed a series of membranes in which the exogenous cyclic AMP-dependent protein kinase stimulated the formation of PIP from PI (8-11). These results led us to the conclusion that this effect of the cAMP dependent protein kinase may be a general phenomenon when the membranes from different animal cell types are phosphorylated under in vitro conditions.

It is not surprising that in those membrane preparations where the lipid phosphorylation was damaged the effect of the protein kinase was not observed. The reason is not known why the membranes prepared from different animals varied so strongly but it is conceivable that in some animals in the course of liver perfusion and cell preparation a small or large part of the phosphatidylinositol pool might be hydrolysed hence it could not be phosphorylated in the isolated membrane, while in other cases the phosphatidylinositol pool was preserved.

A more important question is whether the in vitro effect of the cAMP-dependent protein kinase on lipid phosphorylation has a biological significance or not, since cAMP has been suggested to inhibit the stimulus-dependent hydrolysis of PIP_2 in certain cells while the increase of PIP content of these cells has not been observed (20). We observed the regulatory effect of the cAMP-dependent protein kinase on PIP formation of iso-

lated membranes in the presence of a rather high protein kinase activity. It is known that under in vitro conditions the cAMP-dependent protein kinase is able to phosphorylate a large number of proteins which are not substrates of the enzyme inside the cell. However, the catalytic activity of an artificial substrate is generally not influenced by phosphorylation.

It has been reported that several tyrosin protein kinases (21, 22) and the phosphorylase kinase (23) may also phosphorylate phosphatidylinositol directly. The cAMP-dependent protein kinase is known to recognize basic determinants of primary structure of its substrates around the phosphorylated site (24), hence it is difficult to think that it has a direct effect on phosphatidylinositol. (We could not demonstrate a direct effect.) Nevertheless, in our experiments the protein which could be responsible for lipid phosphorylation and which is regulated by phosporylation has not been identified. Previously we compared the phosphoprotein patterns of several membranes in which the stimulating effect of the cAMP-dependent protein kinase on PI phosphorylation was observed. A 24 kDa protein was found to be the single phosphoprotein present in the membrane of each cell type tested. Therefore we presumed that this protein has been involved in the regulation of lipid phosphorylation (25). Our present study is unable to confirm this hypothesis. A minor 24 kDa phosphoprotein component was observed also in hepatocyte membranes, but the phosphorylation of this protein was not completely inhibited by the heat stable inhibitor protein of the cAMP-dependent protein kinase (Fig. 3).

The subcellular localisation of the cyclic AMP-dependent regulation of PIP formation is also to be clarified by further investigations. Though PIP is generally accepted to be formed in the plasma membrane, we have detected the stimulated formation of PIP in a sarcoplasmic reticulum preparation of heart (11). In hepatocytes a phosphatidylinositol kinase associated with microsomes has been described (26) and the formation of PIP in other membranes besides plasma membrane has also been reported (27). PIP is a powerful activator of the Ca^{2+}-Mg^{2+}-

dependent ATP-ase under conditions when the calcium concentration is lower than 10^{-6} M (Enyedi Á. personal communication). The possible participation of the cAMP-dependent PIP formation in the regulation of the Ca^{2+}-pumping activity of the plasma membrane (or endoplasmic reticulum) at the resting state of the calcium messenger system is an promising hypothesis.

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INSOLUBLE GLYCOGEN AND ITS INTERACTION WITH PHOSPHORYLASE. A NOVEL METHOD FOR THE PURIFICATION OF LIVER PHOSPHORYLASE A

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SUMMARY

Purified liver glycogen dissolved in Tris-HCl buffer (pH 6.8) was converted into an insoluble polymer by incubation with phosphorylase and glucose 1-phosphate. Elongation of the outer chains of glycogen did not alter the average molecular weight significantly as judged by sedimentation velocity measurements, but the spectrophotometric analysis of glycogen-iodine complexes showed marked differences. Insoluble glycogen could bind rabbit skeletal muscle and liver phosphorylases. The association of insoluble glycogen with phosphorylase could be treated as a distribution equilibrium between glycogen-bound and unbound phosphorylase. Based on the formation of an insoluble glycogen protein complex sedimentable even by low-speed centrifugation, a novel method has been developed for the purification of liver phosphorylase a in a homogeneous form.

INTRODUCTION

Glycogen is a readily mobilized fuel-store in the tissues. It can be isolated by high-speed centrifugation from the tissue homogenates as a protein-glycogen complex which is thought to represent a structural and functional unit of the cell (Meyer et al., 1970). The association of glycogen with the enzymes of glycogen metabolism has provided convenient means to

Abbreviations: EDTA, ethylenediaminetetraacetic acid; ME, β-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid

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purify e.g. liver phosphorylase (Appleman et al., 1966), skeletal muscle glycogen synthase (Nimmo et al., 1976), liver phosphorylase kinase (Chrisman et al., 1982) and skeletal muscle phosphorylase phosphatase (Villa-Moruzzi, 1986).

There are two main enzymatic pathways for glycogen synthesis. The synthesis of glycogen from UDP-glucose by glycogen synthase is irreversible and considered to occur in vivo. The synthesis of glycogen from glucose 1-phosphate is also catalyzed by phosphorylase in vitro (Illingworth et al., 1961). High molecular weight glycogen was synthesized with purified phosphorylase and branching enzyme and was similar to the undegraded liver glycogen in molecular weight and appearance under the electron microscope (Mordoh et al., 1965, 1966). Sedimentation velocity, iodine colour, stability of synthetic glycogen against heat and acid treatments, β-amylolysis and sonication have also been studied and compared with those of native glycogen (Illingworth et al., 1961; Mordoh et al., 1965, 1966; Parodi et al., 1967). On the other hand, no data on protein complexes of synthetic glycogen are available at present.

The formation of a highly insoluble glycogen by incubation of a soluble one with purified phosphorylase and glucose 1-phosphate is reported in this paper. The association of insoluble glycogen with phosphorylase has been studied and a novel method for the purification of rabbit liver phosphorylase a has been developed.

MATERIALS AND METHODS

DE-52 was purchased from Whatman (England) and 5'AMP-Sepharose 4B from Pharmacia Fine Chemicals (Sweden). Glycogen (TCA) was isolated from rabbit liver according to Stetten et al. (1956) by extraction with cold TCA. Phosphorylase b was purified from rabbit skeletal muscle as described by Fischer and Krebs (1962).

Buffers used were as follows.

Buffer A: 40 mM Tris-HCl (pH 6.8), 2 mM EDTA, 10 mM ME;
Buffer B: 20 mM sodium β-glycerophosphate (pH 7.0), 5 mM EDTA, 10 mM ME, 50 mM NaF;
Buffer C: 50 mM sodium β-glycerophosphate (pH 7.4), 10 mM ME, 50 mM NaF;
Buffer D: 5 mM Tris-HCl (pH 7.0), 1 mM EDTA, 10 mM ME, 50 mM NaF;
Buffer E: Buffer A containing 2% glycerol.

Preparation of insoluble glycogen

500 mg glycogen (TCA) was incubated with 0.5 mg muscle phosphorylase b at 30°C in 20 ml solution of buffer A containing 50 mM glucose 1-phosphate and 1 mM AMP until no more inorganic phosphate was liberated (10-20 min). Reaction course was also monitored by the change in the colour of glycogen-iodine complex from yellowish brown to violescent. The solution was mixed with 10 ml of 10% TCA and centrifuged at 3000 x g for 10 min at 20°C. Then 1.5 vol. of 96% ethanol was added to the supernatant and it was allowed to stand for 1 hour at 0-4°C. The precipitated glycogen was decanted and centrifuged as before. The supernatant was discarded and the pellet was dissolved in 20 ml buffer A at room temperature. If necessary, the centrifugation was repeated to remove any remaining precipitate. After overnight storage at 0-4°C a highly insoluble glycogen was precipitated from the clear supernatant and could be collected by centrifugation at 1000-2000 x g.

The product of this step, termed as insoluble glycogen, could not be dissolved in water or buffer A even at 30°C, only in 1 M NaOH. Insoluble glycogen used for the purification of phosphorylase a was stored in buffer B.

Assay of phosphorylase activity

Muscle phosphorylase b activity was assayed according to Illingworth and Cori (1953). Liver phosphorylase a activity was assayed by the method of Stalmans and Hers (1975). The incorporation of the glucosyl moiety of glucose 1-phosphate into glycogen was followed by the release of inorganic phosphate (Taussky and Shorr, 1953).

Purification of rabbit liver phosphorylase a

Rabbit was fasted for 48 h and anaesthetized with Inactin (100 mg/kg). Glucagon (100 µg/kg) was injected intravenously 15 sec before cutting the jugular veins. The liver was removed and homogenized in 3 volumes of ice-cold buffer B containing 10 mM benzamidine-HCl and 0.5 mM PMSF. All subsequent steps were carried out at 0-4°C. The extract was centrifuged at 8000 x g for 30 min. The supernatant was filtered through glass wool.

100 mg insoluble glycogen in a suspension of 1 ml was added to the homogenate and stirred for 10 min. Glycogen particles were isolated by centrifugation at 5000 x g for 5 min. The supernatant was mixed with another 100 mg portion of insoluble glycogen and centrifuged as before. Insoluble glycogen pellets were combined and 10 ml of buffer B was added to decrease the amount of contaminating proteins co-sedimented with the glycogen-protein complex. After mixing the suspension was centrifuged and the pellet was washed once more with buffer B.

Phosphorylase a bound to glycogen was eluted by the procedure of Hwang et al. (1984) with the following modifications. Glycogen-protein complex was homogenized in 15 ml of buffer C containing 30% (w/v) maltose and stirred for 30 min. After centrifugation the elution procedure was repeated with the pellet. Supernatants were combined and dialyzed for 24 h against three changes of 1000 ml buffer D containing 10% (v/v) glycerol.

The dialysate was loaded on a 3 x 10 cm DE-52 column, equilibrated with buffer D. The column was washed with several volumes of buffer D and phosphorylase a was eluted with a 300 ml linear gradient of 0-0.3 M NaCl in buffer D. Fractions containing phosphorylase a activity were pooled and dialyzed against buffer E (3 x 5 volumes). The sample was concentrated to 2-3 ml with an Amicon concentrator using a PM-30 membrane.

The enzyme solution was applied to an AMP-Sepharose column (1.5 x 5.0 cm) with was equilibrated with buffer E. Unadsorbed proteins were washed out with buffer E and phosphorylase a was eluted with 10 mM AMP dissolved in buffer B. Active fractions were collected, dialyzed against buffer E (3 x 200 ml) and concentrated as before. The preparation was stored in the presence of 50% glycerol at -10°C.

Other methods

Spectrophotometric analysis of the glycogen-iodine complexes was carried out in a CE 595 double-beam, scanning spectrophotometer, against an iodine/iodide reference solution similar to Krisman (1962).

Sedimentation velocity experiments were performed at 20°C using a MOM 3180 analytical ultracentrifuge with a Schlieren optical system. After attaining rotor speed of 20 000 rpm, exposures were made in 4 min intervals. Sedimentation patterns were analyzed with a microcomparator.

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) in 7.5% separating gel.

Protein was determined as described by Bradford (1976).

Glycogen was determined according to Krisman (1962) and by the use of anthrone (Hassid et al., 1957).

RESULTS

Properties of insoluble glycogen

Elongation of the side-chains of glycogen (TCA)* resulted in a modified glycogen which was spontaneously precipitated upon standing at 0-4°C, yielding a highly insoluble preparation. Sedimentation velocity experiments were carried out with fresh solutions of the two glycogens (Fig. 1).

Sedimentation coefficient determined at 20°C for glycogen (TCA) was in good agreement with that of obtained by Orrell and Bueding (1964). Very moderate change in the sedimentation behaviour was found after elongating the outer chains of the polysaccharide, suggesting that its average molecular weight had not been increased considerably.

*in the presence of glucose 1-phosphate and phosphorylase

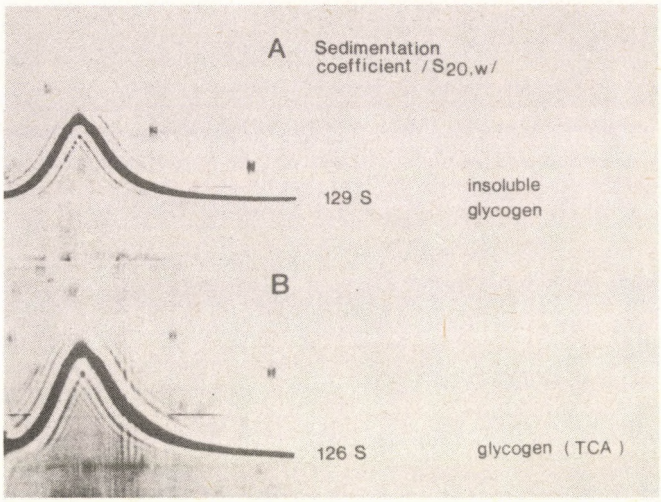

Fig. 1. Sedimentation velocity patterns of insoluble glycogen: (A) and the original glycogen (TCA): (B) at 20°C. 10 mg/ml glycogen solutions were applied in the experiments as described in Materials and Methods. The pictures were taken 12 min after the rotor had reached 20 000 rpm.

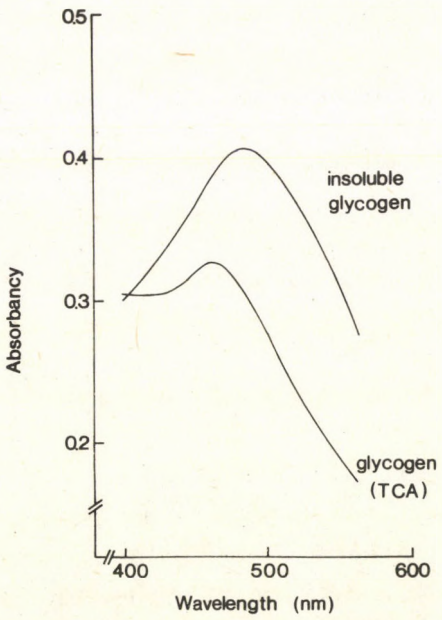

Fig. 2. Absorption spectra of iodine complexes of glycogen (TCA) and insoluble glycogen.

Spectral analysis of glycogen-iodine complexes in the presence of saturated $CaCl_2$ was carried out on glycogen solutions of 0.2 mg/ml (as determined by anthrone). As seen in Fig. 2., the maximum absorption was shifted to longer wavelength and absorbancy increased following chain-elongation.

Binding of phosphorylase to insoluble glycogen

Insoluble glycogen was capable of complexing with phosphorylase in crude rabbit skeletal muscle extract (not documented). Binding was studied using purified rabbit muscle phosphorylase b (Fig. 3).

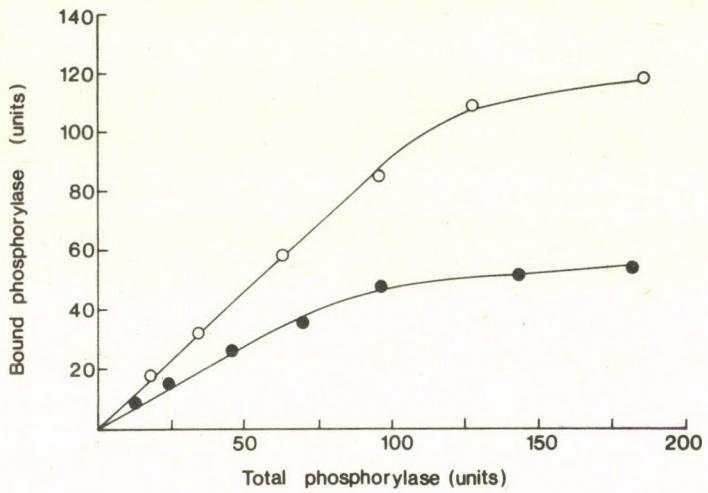

Fig. 3. Binding of phosphorylase b to insoluble glycogen at 0°C. 5 mg insoluble glycogen was applied in 3 ml (o) and 30 ml (●) buffer A, respectively.

The initial part of both curves is almost linear and demonstrates a distribution equilibrium between the insoluble glycogenic and soluble phases. The ratio of phosphorylase b concentration bound to insoluble glycogen and that of remained in solution was calculated in both cases and proved to be constant, approx. 10^4 with a standard deviation of $\pm$20%. (The density of

glycogen was taken as 1 g/cm^3 in these calculations.) Insoluble glycogen could be saturated by increasing the amount of phosphorylase b in the suspension of a given volume. The maximum adsorption capacity of glycogen was strongly dependent on the volume of suspension (i.e. on the enzyme concentration).

Table 1. Elution of phosphorylase b from insoluble glycogen. 30 units of phosphorylase b bound to 4 mg glycogen were handled with 2 ml solutions for 10 min at 0°C.

Eluent	Phosphorylase b eluted (%)
Buffer A	5
Buffer B	3
30% glucose	49
30% sucrose	6
30% lactose	4
30% maltose	85
1% glycogen (TCA)	69
1% glycogen (TCA) at 30°C	100

In order to apply insoluble glycogen for purification of the enzyme, bound phosphorylase must be liberated. As shown in Table 1, no elution was obtained with buffers containing β-glycerophosphate or Tris. Glucose was able to translocate the enzyme to a certain extent. Maltose could remove the greater part of the bound enzyme, two other disaccharides were without significant effect. On the other hand, glycogen (TCA) could elute phosphorylase b in a solution of 1%. Since the eluent must be removed in the course of an isolation procedure, maltose was chosen as it is dialyzable.

Purification of phosphorylase a from rabbit liver

Liver phosphorylase makes up only a small fraction of the total soluble protein (Hwang et al., 1984), therefore its purification is more difficult than that of the muscle enzyme. By

the introduction of insoluble glycogen for the preparation of liver phosphorylase a simplified procedure has been developed. Glycogen of rabbit liver was depleted and phosphorylase was activated in response to fasting and glucagon treatment. The inactivation (dephosphorylation) of phosphorylase a was inhibited by NaF throughout the preparation.

Table 2. Purification of phosphorylase a from rabbit liver

Step	Protein	Phosphorylase a		Purification	Recovery
	(mg)	Activity (units)	Spec.act. (units/mg)	(fold)	(%)
Homogenate	7498	994	0.13	1	-
Insoluble protein-glycogen complex	201	654	3.25	25	100
Washed protein-glycogen complex	72	628	8.72	67	96
Maltose eluate	70	602	8.60	66	92
DE-52 chromatography	11.5	255	22.10	170	39
AMP-Sepharose chromatography	7.5	190	25.30	195	29

Table 2 summarizes the purification steps, using 60 g of rabbit liver as starting material. 200 mg of insoluble glycogen was used in two equal portions. The previous experiments showed a distribution equilibrium between glycogen-bound and free phosphorylase in liver extracts similar to that observed with the purified muscle enzyme but the distribution ratio was 5-8 times lower (not documented). Equilibrium was approached within 10 min. The resulting insoluble protein-glycogen complex could be isolated by low-speed centrifugation for 5 min. Specific activity of the bound enzyme was greatly enhanced by washing it, without a considerable loss. The effect of maltose to trans-

locate the enzyme may be at least in part due to its maltotriose contamination, which is a competing ligand for the glycogen site of phosphorylase (Kasvinsky et al., 1978).

Phosphorylase a obtained after ion-exchange and affinity chromatography was homogeneous as judged by polyacrylamide gel electrophoresis (Fig. 4). The subunit molecular weight was around 94 kDa similar to that of the crystalline rabbit muscle phosphorylase b.

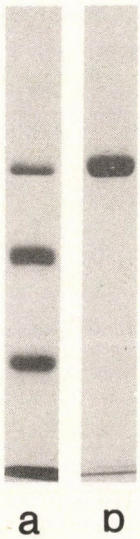

Fig. 4. SDS gel electrophoresis of purified liver phosphorylase a. Samples were run on a 7.5% SDS-polyacrylamide gel and stained with Coomassie blue.

(a) molecular mass standards: rabbit muscle phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa.

(b) purified rabbit liver phosphorylase a.

DISCUSSION

The outer chains of glycogen (TCA) were elongated *in vitro* by incubation with purified phosphorylase and glucose 1-phosphate. This enzyme is known to catalyze glycogen synthesis

in vitro, but some histochemical studies suggest it might have also a role in the in vivo synthesis in exceptional cases (Amemiya, 1978; Amemiya, Yoshida, 1980). In spite of an increase in the total mass of glycogen in the reaction mixture, only slight change was detected in the sedimentation coefficient indicating no considerable increase in the average molecular weight.

The light absorption maximum of the glycogen-iodine complex was shifted toward the higher wavelengths by elongating the side-chains of glycogen, in agreement with the results of Illingworth et al. (1961) obtained with synthetic glycogen. Our data support of those of Krisman (1962) that the exterior chains of glycogen may have a role in the colour formation.

Glycogen modified in vitro by our procedure was also sedimented by low centrifugal forces, like "overgrown molecules" prepared by Mordoh et al. (1965) in the presence of phosphorylase and branching enzyme.

"Soluble" glycogen is well-known to complex with phosphorylase and other enzymes involved in glycogen metabolism. Glycogen and its analogues prefer the binding to the glycogen storage site, but they can also bind to the catalytic site of the enzyme (Kasvinsky et al., 1978). The enzyme-binding site of the glycogen molecule is suggested to participate in the complex formation (Bresler and Firsov, 1971).

We found insoluble glycogen is also capable of complexing with phosphorylase. In cases of low enzyme concentration, the amount of bound phosphorylase is determined by the distribution law. Increasing the enzyme concentration in the suspension, insoluble glycogen can be saturated. Klinov et al. (1982) reported that the maximum adsorption capacity of pig liver glycogen was 3.64 µmol dimeric phosphorylase b per g glycogen. From the data of Fig. 3 (upper curve) one can calculate a value of 2.6 µmol dimeric phosphorylase b per g insoluble glycogen, although it is not easy to compare those for the above reason.

Insoluble glycogen resembles amylose in its side-chains. Starch solution has been used for the purification of potato (Kamogawa et al., 1968) and spinach phosphorylases (Shimomura

et al., 1982), furthermore starch column has been developed for the purification of heart phosphorylases (Hanabusa and Kohno, 1969). On the other hand, in our preliminary experiments insoluble glycogen had a much higher phosphorylase binding capacity than starch under the same conditions (G. Bot, unpublished observation).

Protein-glycogen complex obtained by high-speed centrifugation is generally used as a source for the purification of liver phosphorylase. The preparative scheme usually includes one or more steps of DEAE column chromatography (Appleman et al., 1966; Maddaiah and Madsen, 1966; Wolf et al., 1970; Stalmans and Hers, 1975; Hwang et al., 1984), AMP or hydrocarbon-linked Sepharoses (Kobayashi and Graves, 1982; Livanova et al., 1976) or isoelectric focusing (Livanova et al., 1976). The majority of these methods yields phosphorylase in the catalytically inactive b form. To obtain phosphorylase a *in vitro* phosphorylation with phosphorylase kinase has to be performed.

Our novel method is based on the complex formation between phosphorylase a phosphorylated *in vivo* and insoluble glycogen. The distribution equilibrium between bound and free phosphorylase a is approached within 10 min, and the complex can be sedimented by low-speed centrifugation for 5 min instead of high-speed centrifugation for 2-3 h applied in the procedures above. Thus the preparation is much more rapid, the proteolysis of phosphorylase during this step could be avoided. By addition of more insoluble glycogen to the homogenate, the amount of bound phosphorylase can only be enhanced to an extent determined by the distribution ratio. Our elution step with maltose is based on the method of Hwang et al. (1984). To obtain phosphorylase a showing a single band on SDS-polyacrylamide gel, the enzyme has to be purified on AMP-Sepharose column following ion-exchange chromatography, although it gives only a slight improvement in the specific activity. Our method seems to be suitable for the purification of phosphorylase in cases when tissue glycogen has been depleted.

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STUDY OF TRANSPORT PROCESSES IN SOILS AND PLANTS BY MICROAUTORADIOGRAPHIC AND RADIOABSORPTION METHODS

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SUMMARY

The concentration profiles of lead and boron in carrot root and potato tuber were determined at various diffusion times by microradiographic method. The order of magnitude of the diffusion coefficient of lead ions and borate- or tetraborate ions was 10^{-13} m^2s^{-1} and 10^{-11} m^2s^{-1}, respectively.

The transport process of nutrients, leaf-manures and plant-protecting agents in plants was investigated by radioabsorption method. The effect of complex-forming agents on the absorption of nutrients and leaf-manure compositions was also examined.

The influence of the pH of soils and complex-forming agents on the effective diffusion coefficients of nutritives was studied by means of the radioabsorption technique. In soils, the effective diffusion coefficient of the nutrients was found to change in the region 10^{-16}-10^{-10} m^2s^{-1}.

The data of the measurements give valuable information about the transport processes in plants and soils, allowing control and influence thereof.

INTRODUCTION

The transport processes proceeding in soils and plants decisively determine the ability of the soil for nutriment transport and the corp yields of plants.

The subjects of our work were monitoring the material transport proceeding in soils, as well as the investigation of the passive material transport process in palnts carrying the nutrients to the cells and to the delivery system through the internal covering tissues and intracellular ducts. These mate-

rial transport processes are decisively determined by three factors: diffusion, sorption and circulation, according to the following relationship (Lapidus and Amundson, 1952):

$$\frac{dc}{dt} = D' \frac{d^2c}{dx^2} - \frac{m}{w}\frac{da}{dt} - u' \frac{dc}{dx} \qquad (1)$$

where c represents the concentration of the nutrient at time t and at distance x from the reference surface; D' is the diffusion coefficient of the material considered; u' is the rate of the material transport; w is the porosity and m is the specific density of soil or plant and a is the specific amount of the sorbed nutrient in terms of g/g. If the sorption process can be described by a linear isotherm, we can introduce the q distribution coefficient:

$$q = \frac{m}{w}\frac{a}{c} \qquad (2)$$

and thus equation (1) can be simplified (Jost, 1953) as

$$\frac{dc}{dt} = D \frac{d^2c}{dx^2} - u \frac{dc}{dx} \qquad (3)$$

where $D = \frac{D'}{1+q}$ and $u = \frac{u'}{1+q}$.

The solution of equation (3) for our experimental conditions is given in the Methods.

The main part of the methods applied recently gives only quantitative information on the transport processes, even in summarizing works (Lüttge and Higinbotham, 1979; Höfer, 1981; Bolt, 1979; Sparks, 1986) only few quantitative data are available. Therefore, we have developed and used methods which are suitable for the quantitative determination of the parameters of transport processes in plants and soils.

METHODS

For studying the passive material transport in plants quantitative microautoradiographic and radioabsorption methods were applied. For the measurements aimed at the determination of the transport processes in soils the radioabsorption method was used. The methods applied for monitoring the material transport processes in plants or in soils are based on the use of radioactive tracers or nuclear reactions induced on stable nuclei.

Quantitative microautoradiography

For the measurement of the transport processes in plants the following three methods were applied: photoemulsion-autoradiography, alpha-autoradiography performed by solid-state track detectors and neutron-induced autoradiography. The applied methodological steps for the latest method are shown in Fig. 1.

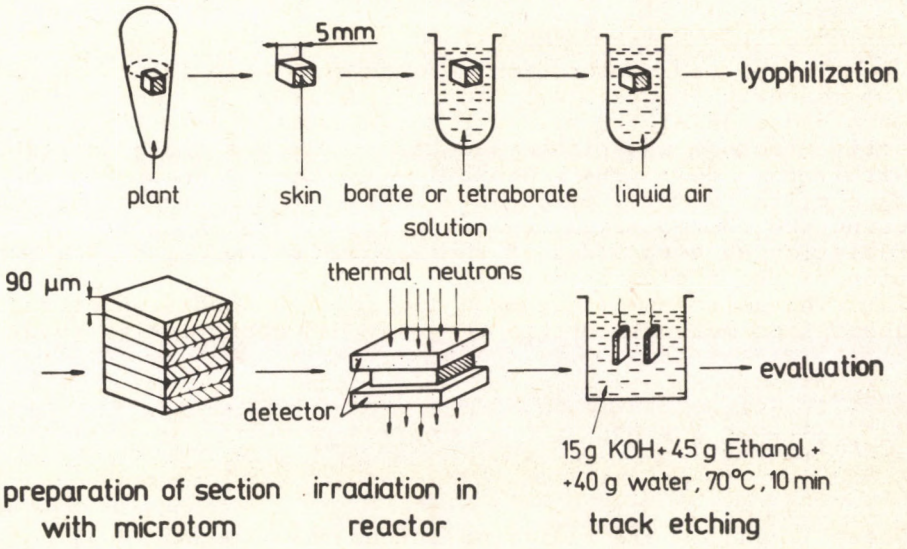

Fig. 1. Methodological steps applied for studying the boron transport in plants.

By using quantitative microautoradiography a sample of the plant was placed in a solution containing either the radioactive isotope or the stable nuclide in case of the induced autoradiography. Experimental conditions were such as to ensure only a negligible change (1-2%) in the concentration of the solution during this procedure. After a required period of contact the sample was removed from the thermostated device, the transport process was frozen with liquid air and the sample

was lyophilized. The sample was then embedded in bee wax, cut into thin slices with microtome and the slices were placed on the detector. After the required time of exposure the radiograms were developed and evaluated.

For the evaluation of concentration profile of isotopes the solution of equation (3) - determined by the actual experimental conditions - was applied (Crank, 1956)

$$c = c_o \operatorname{erfc} \left(\frac{x - ut}{2\sqrt{Dt}} \right) \tag{4}$$

where c_o represents the isotope concentration measured in the surface layer of sample.

$$\left[\frac{x - ut}{2\sqrt{Dt}} = y; \quad \operatorname{erfc} y = 1 - \frac{2}{\sqrt{\pi}} \int_o^y e^{-y^2} dy \right] .$$

Radioabsorption technique

In the radioabsorption measurements a device equipped with two measuring heads developed in our laboratory was applied. Onto one side of the plant or soil sample a β-emitting radioactive isotope was placed and the sample was closed airtight with a thin foil. The radioactive isotope, placed onto the surface of the sample, penetrates increasingly into the sample. By using the thermostated measuring system the intensity of the radiation at both sides of the sample was measured as a function of the diffusion time.

The solution of equation (3) for the condition of radioabsorption measurements is (Krjukov and Zhuhovickij, 1953):

$$\frac{I_1 - I_2}{I_1 + I_2} = K \exp\left(-\pi^2 D_{eff}\, t/L^2\right) \tag{5}$$

where I_1 and I_2 are radiation intensities, measured at both sides of the sample, K is a constant, the value of which is dependent on the β-ray absorption coefficient of the sample, D_{eff} is the effective diffusion coefficient of tracer, involving the overall effect of diffusion, sorption and material transport and L is the thickness of the sample.

RESULTS AND DISCUSSION

Autoradiographical measurements in plants

The passive transport of lead, known as a plant poison and environmental pollutant, was investigated in carrot root by

autoradiography. Cubiform samples of 5 mm edge length were cut from carrot root so that one face of the samples was formed from the skin-part of the root. The concentration distribution of the α-activity of ^{212}Pb isotope inside the plant was determined by both photoemulsion and solid-state track detector techniques. For the evaluation of concentration profiles the equation (4) was applied, and the transport parameters derived are listed in Table 1.

Table 1. Diffusion coefficient and flow rate of lead in carrot root measured at various temperatures and diffusion times with 34-B-50 Agfa-Gevaert photoemulsion

Diffusion time (min)	Temperature (°C)	Flow rate (10^{-9} ms^{-1})	Diffusion coefficient (10^{-13} m^{2}s^{-1})	
			In internal tissue	In external skin tissue
10	25	6.3±3.5	2.8±1.2	3.5±1.3
30		5.5±3.1	2.7±0.7	3.2±0.7
60		4.4±3.0	2.3±0.5	2.5±0.7
120		5.5±3.8	2.7±0.8	2.6±0.9
60	0	3.1±2.5	1.2±0.4	1.5±0.4
	25	4.4±3.0	2.3±0.5	2.5±0.7
	40	6.2±3.6	4.9±1.9	4.5±0.7

The order of magnitude of the material flow-rate and diffusion coefficient are 10^{-9} ms^{-1} and 10^{-13} m^{2}s^{-1}, respectively. The values obtained for the diffusion coefficient by means of the photoemulsion and solid-state track detector techniques are in good agreement.

The transport process of boron, one of the most important microelements, was investigated in carrot root and potato tuber in the form of borate and tetraborate ions by means of neutron-induced autoradiography technique.

The original boron content of the samples was 30-50 mg/kg of dry material as determined with a Zeiss Q-24 quartz spectrograph by using the emission method.

The plant samples were placed into 10 ml of 0.1 mol dm^{-3} boric acid or sodium tetraborate solution and then the methodological steps shown in Fig. 1 were applied. Onto both sides of the plant sections 200 μm thick Makrofol-E polycarbonate track detectors were mounted, and the samples were irradiated with a thermal neutron fluence of $2 \cdot 10^{11}$ neutron$\cdot cm^{-2}$ in the nuclear reactor of Ljubljana using a cadmium ratio of 40. The value of the thermal neutron flux at the place of irradiation was 10^{8} neutron$\cdot cm^{-2} \cdot s^{-1}$ and the time of irradiation was about 33 minutes.

For the development of the tracks of α-particles and ^{7}Li nuclei produced in the $^{10}B(n,\alpha)^{7}Li$ nuclear reaction a mixture of 15 g KOH + 40 g ethanol + 45 g water was used (etching time: 10 min. at 70°C) (Somogyi and Gulyás, 1972).

The boron concentration profile inside the plant was mapped on the track detector by etch-track cones of 1-2 μm in diameter and its typical shape measured in carrot root is shown in Fig.2.

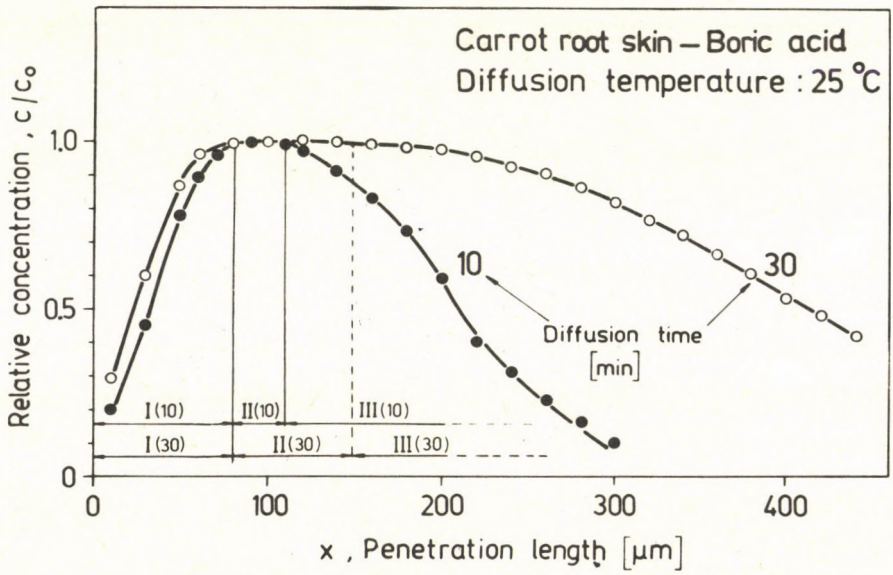

Fig. 2. Distribution of boron concentration in the skin tissues of carrot root.

The profile corresponding to the distribution of the boron concentration inside the plant can be divided into three relatively well-distinguished parts.

i) In the first part, on proceeding from the surface of the plant towards its interior, the concentration progressively increases up to a limit value. The lenght of this part varies in the range of 20-80 μm, and seems to be independent of the diffusion time, but it is determined by the surface inhomogenities. This penetration zone developes immediately when the plant and the solution comes into contact, thus in the calculation of transport parameters this is regarded as the starting state.

ii) In the second part the concentration is constant and the length of this increases as the diffusion time increases. From this data the rate of material flow may be calculated.

iii) The third part is the so-called diffusion zone, where the distribution of material concentration, with regard to the position and time, can be expressed by equation (4).

The values of diffusion coefficients determined by neutron-induced autoradiography are summarized in Table 2. The indicated values are calculated from the average data of 15-20 measurements. The standard deviation of these measurements is about ±30-40%.

The order of magnitude of the diffusion coefficients characteristic of the transport of boron in carrot root and potato tuber is 10^{-11} m^2s^{-1}. The transport of boron is faster than that of the tetraborate ion and the value of the diffusion coefficient is about twice as large in the internal tissues than that in the external skin tissues.

The above microautoradiographic measurements are regarded as model experiments to demonstrate the capability of our method for mapping the ion concentration profiles in plants, and for the quantitative determination of the transport parameters of nutrients.

Radioabsorption measurements in plant samples

From the parenchima tissues of different plants disk-shaped samples of 1 cm diameter were prepared. The thickness

Table 2. Diffusion coefficients of borate and tetraborate ions in carrot root and potato tuber at 25°C

Plant	Penetrating ion	Diffusion time (min)	Diffusion coefficient (10^{-11} m^2s^{-1})	
			In external skin tissue	In internal tissue
Carrot root	Borate	5	1.9±0.4	1.8±0.5
		10	1.7±0.7	1.6±0.5
		30	1.7±0.6	1.8±1.0
	Tetra-borate	5	1.0±0.3	2.1±0.9
		10	0.9±0.2	1.9±0.7
		20	1.0±0.2	1.5±0.4
Potato tuber	Borate	10	2.3±0.6	5.7±2.4
		20	2.4±0.5	4.1±1.9
		30	2.1±0.5	4.9±2.3
	Tetra-borate	5	1.1±0.6	2.9±1.1
		10	1.4±0.6	3.3±1.7
		20	1.2±0.4	2.3±0.9

of samples was 1-3 mm and that of leaves ranged between 80-150 μm. The effective diffusion coefficients were determined from 3-5 parallel measurements with a standard deviation of about 20-30%.

In Figure 3. the effective diffusion coefficients measured for some nutrients are indicated in the presence and in the absence of complex-forming agents. It has been found that the rate of absorption of the nutrients in plants can be significantly accelerated by the addition of complex-forming materials. The most effective compositions and the optimum concentrations of the complex-forming materials may be selected by such measurements.

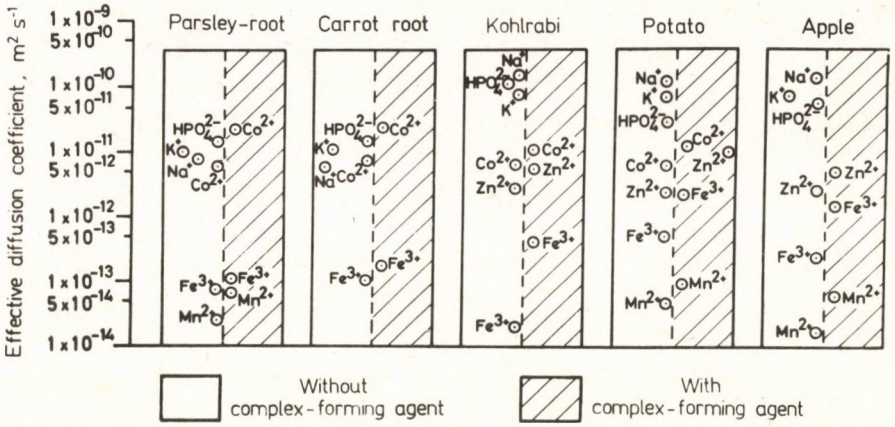

Fig. 3. Effective diffusion coefficients characteristic of the transport of some nutrients at 25°C. The complex-forming material used is the oxidized product of saccharose at a concentration of 0.1 mol·dm^{-3} (Beck et al., 1979, Hungarian Patent).

Having improved the radioabsorption method an equipment was developed for the measurement of the transport processes of compounds labelled with soft β-emitting isotopes. This allowed the determination of the transport of plant protecting agents in various plants. With this equipment we could study the transport processes of compounds labelled with ^{14}C and ^{3}T, or with other soft β-emitting isotopes (Varró et al., 1984, Hungarian Patent).

The absorption of ^{14}C-labelled Saphidon (o,o-dimethyl-mercaptomethyl-phthalimido-dithiophosphate) was studied in different plant leaves and membranes. Effective diffusion coefficients typical for the Saphidon-emulsion are summarized in Table 3.

The radioabsorption measurements provided important information about the formation and the quality of formation of plant protecting agents. Such measurements allow the selection of the appropritate solvents and emulgators, and also a quick determination of the optimal compositions.

The equipment developed for studying the transport processes of different compounds labelled with soft β-emitting isotopes allows the determination of the transport of biologi-

Table 3. Effective diffusion coefficients characteristic of the transport of Saphidon-emulsion in plant membranes at 25°C.

The composition of the emulsion is 15% Saphidon + 25% Xylene + 2% Dimethyl sulphuroxide + 3% Acetone + 5% Emulgator (a 2:1 mixture of Triton-X-80 and Triton X-180) + 50% Water.

Leaves	D_{eff} $[10^{-15} m^2 s^{-1}]$	Fruit peel	D_{eff} $[10^{-15} m^2 s^{-1}]$	Vegetable skin	D_{eff} $[10^{-15} m^2 s^{-1}]$
Maize	15	Plum	1.8	Tomato	3.3
Potato	52	Pear	175	Potato	9.2
Acacia	23	Grape	207	Onion	83
Pansi	350	Peach	240	Parsley	290
				Carrot	350

cally active compounds in plant membranes with high sensitivity. Our method permits a fast and well-reproducible determination of the diffusion coefficients in the interval of 10^{-9}-$10^{-17} m^2 s^{-1}$ with a standard deviation of ±20-30%.

Studies on the transport processes of nutrients in soils by radioabsorption method

By using β-emitting isotopes the radioabsorption method allows the determination of the transport processes of the most important nutrients and microelements in cultivated lands. Some characteristics of soils studied from this point of view are given in Table 4. The effective diffusion coefficients measured for some nutrients in soils are summarized in Table 5.

The values of the effective diffusion coefficient in various soils varied in the region of 10^{-16}-10^{-10} $m^2 s^{-1}$. The transport of the cations was the highest in sandy soil and the lowest in loamy and alkalised soils, whereas the diffusion coefficient of the HPO_4^{2-} ions varied in the opposite way. One can see that variations in the soil structure considerably influence the transport of nutrients. The absorption parameters of the nutrients in soils can also be significantly changed by the appli-

cation of additive agents, for example complex-forming materials.

Table 4. Characteristics of the soil samples studied

Parameter	Type of soil				
	Sandy soil	Loamy soil	Clay loam	Clay soil	Alkalised soil
Restriction number	25	40	52	55	55
pH (in H_2O)	7.7	7.9	5.8	6.6	9.6
pH (in KCl)	7.3	6.9	5.1	5.9	8.2
Humus (%)	0.25	2.8	3.2	3.1	0.75
Assumable P_2O_5 (mg/100 g)	5.4	27.1	11.1	32.7	33.7
Assumable K_2O (mg/100 g)	9.3	36.5	50	50	27.3
Mechanical composition (%) <0.001 mm	5.8	26.6	37.0	47.4	32.6
0.001-0.01 mm	3.2	18.0	25.2	22.2	16.2
0.01-0.25 mm	91.0	55.4	37.8	30.4	51.2
Capillary water-raising in 5 hrs (mm)	350	125	45	28	0

The effective diffusion coefficients of various nutrients measured in different soil samples, in the presence and absence of a complex-forming agent are shown in Fig. 4.

The transport processes of nutrients in soils can be sensitively followed by radioabsorption method, and a quick and detailed examination of both the process itself and the parameters influencing thereof can be performed in a wide measuring range. The results of such measurements give valuable quanti-

Table 5. Effective diffusion coefficients characteristic of the ion transport process in soils of 37.5% humidity at 25°C

Ion	Effective diffusion coefficient (10^{-14} m^2 s^{-1})				
	Sandy soil	Loam soil	Clay-loam	Clay-soil	Alkalised soil
Na^+	18 000	10 000	12 000	11 500	9 100
K^+	8 500	3 500	3 500	3 500	3 100
Cs^+	360	270	150	250	280
Ca^{2+}	4 100	630	900	530	250
Mn^{2+}	630	280	730	420	190
Co^{2+}	190	110	100	130	79
Zn^{2+}	2 500	1 000	93	1 100	30
Fe^{3+}	0.72	0.04	0.27	0.17	0.11
HPO_4^{-2}	19	830	18	24	180

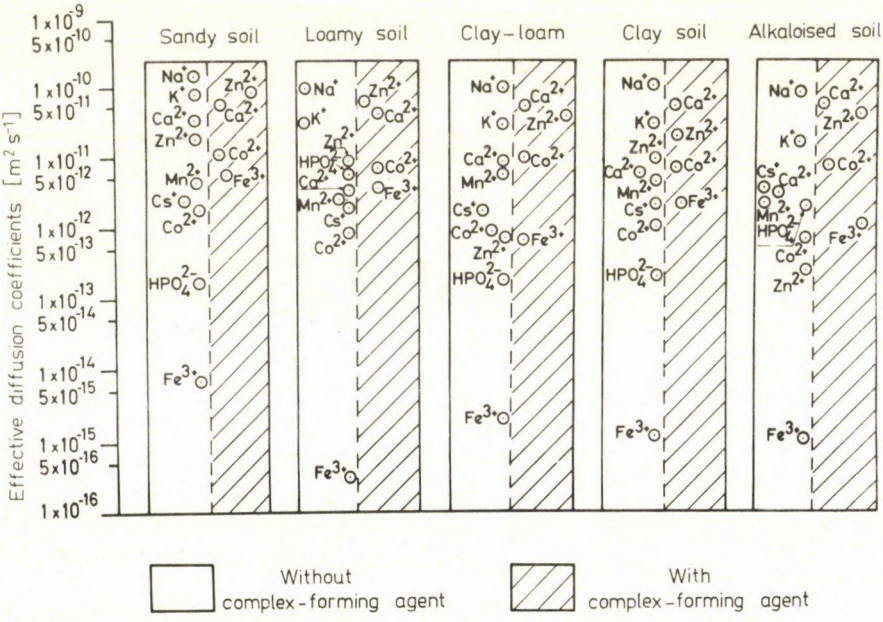

Fig. 4. Effective diffusion coefficients of nutrients measured in various soils at 25°C and 37.5% soil humidity. The complex-forming agent used was an oxidized product of saccharose at a concentration of 0.1 mol·dm^3.

tative information for the agricultural specialists.

The data obtained by the above radioabsorption measurements refer to the possibilities which can be utilized for influencing and controlling the transport processes of nutrients in different soils and plants.

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FLOW CYTOMETRIC DETERMINATION OF THE SPERM CELL NUMBER IN DILUTED BULL SEMEN SAMPLES BY DNA STAINING METHOD

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SUMMARY

Flow cytometric determination of the number of bull sperm cells showed that the number of spermatozoa measured by light scattering may considerably differ from the actual number of sperm cells in the samples, depending on the proportion of contaminating particles, similar in size to sperm cells. No accurate information can be obtained from the sum of live and dead cells distinguished by means of double fluorescence staining, since a part of the sperm cell population is in a transitory state i.e. between the viable and dead states, so it cannot be stained by either dye.
The number of spermatozoa in the sample can be determined very accurately if the sample is treated first with Nonidet-P-40 detergent then stained with propidium iodide. With this procedure the DNA content of each cell nucleus can be labeled and, through detecting the fluorescence signals, the actual sperm cell count of the sample can be determined with the accuracy of 95-98%.

INTRODUCTION

Initial investigations on male reproductive cells were directed primarily to the morphological and biological properties of the sperm cell. Research work, in the course of time, has become inseparable from economic aspects i.e. from semen production, artificial insemination, actual breeding and in-

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crease in productivity (1-6). Therefore a great number of research projects have been concerned with the objective rating of the semen including the elaboration laboratory tests that are suitable to predict its biological value. Besides the classical microscopic procedures there are, at present, available methods such as electron microscopy (7, 8), enzyme assays (9, 10), laser techniques (11, 12), which allow the precise monitoring of qualitative changes i.e. morphological,physiological, biochemical characteristics of sperm cells. In the semen cells of different physiological quality are present simultaneously and their number and relative proportion is decisive for fertilization. For the determination of the distribution of the particular traits in a population, and the exact definition of the proportions, flow cytometry appears to be a highly efficient method (13-16). The integrity of cell membrane can be checked applying fluorescein diacetate (FDA) and propidium iodide (PI) (12, 17). FDA, which is a nonfluorescent, lipophylic molecule, easily penetrates the cell membrane. The nonspecific intramembrane and intracellular esterases hydrolyse FDA, however only the cells having intact membrane accumulate fluorescein. Whereas PI is taken up exclusively by the dead cells. Applying both fluorescent dyes simultaneously the proportions of live and dead cells can be determined in a flow cytometer.

Our aim in using flow cytometric analysis was to elaborate an objective method for the determination of the cell count in fresh-, equilibrated- and deep-frozen semen samples. In the present paper an account is given on the determination of sperm cell number through studying objective physical parameters.

MATERIALS AND METHODS

Materials

Tris (hydroxymethyl) aminomethane (TRIS), fructose, fluorescein diacetate (FDA), propidium iodide (PI), nonidet-P-40 (NP-40) and RPMI 1640 Medium were obtained from Sigma Chemical Co. (St. Louis, MO. USA). Citric acid, acetone and all inorganic reagents were products of Merck (Darmstadt, FRG).

Preparation of spermatozoa samples

Shortly after ejaculation the semen was diluted 5-10 fold with either TRIS-egg yolk buffer (0.2 M TRIS; 0.077 M citric acid; 0.066 M fructose; 20% vol. egg yolk; pH: 6.8) (fresh samples) or with TRIS-egg yolk buffer containing 10% glycerol and stored at 4°C for 2-20 hours to allow sperm cells to equilibrate with glycerol before freezing (equilibrated samples). The equilibrated semen samples were frozen in straws using the standard freezing procedure and stored in liquid nitrogen (frozen samples). Immediately before examination fresh, equilibrated and frozen samples were diluted with RPMI 1640 (pH: 6.8) to set the cell concentration to 0.5-2 x 10^6 cells/ml necessary for the analysis.

Determination of sperm cell number with Fluorescence Activated Cell Sorter (FACS)

Frequency distribution histograms of fluorescence and light scatter parameters were obtained by using a modified Becton-Dickinson FACS-III flow cytometer as described earlier (18). 0.5-1 x 10^5 cells were analysed in each sample at the speed of about 1 500 cells/s. The volume of the analysed sample was determined by weighing befor and after flow cytometry analysis with accuracy of 0.1 mg.

The following methods were used to determine the actual cell number:

1. The cell number in the first series of the investigation was determined by light scatter measurements. The intensity of scattered light is proportional to the cross-section of objects. Particles having scattered light intensity above a certain threshold were considered as sperm cells.

2. The live and dead cells were specifically labeled by double fluorescence staining (FDA and PI) and the sum of the counted populations i.e. the sum of live and dead ones amounted to the whole cell population present in the sample (12). The possibilities of the simultaneous application of the fluorescent dyes, FDA and PI, have already been described in details (12, 17, 19). In order to label the living cells, FDA was applied in a final concentration of 1 µM and the dead ones were stained with 45 µM PI. The FDA dissolved in acetone was further diluted in PBS (137 mM NaCl, 2,7 mM KCl, 8.7 mM Na_2HPO_4, pH 6.8). Flow cytometric measurements were performed after 8 min incubation of the sperm cells with the dyes at room temperature. Fluorescein emission was detected through an interference filter of 540 nm, PI fluorescence through a 620 cut-off (high pass) filter. For excitation the argon laser was tuned to 488 nm.

3. The cytoplasmic membrane of the cells in the samples was made penetrable for PI through a treatment with detergent. The samples diluted with RPMI 1640 in appropriate aliquots were cooled on ice, then treated with an 1% NP-40 detergent solution. After the treatment with detergent the samples were stained with 45 µM PI. Fluorescence intensity data recorded in the flow cytometer can be directly assigned to the number of cell nuclei.

The cell populations separated by the particular methods were sorted and control measurements were carried out with fluorescence and/or phase-contrast microscopy following the flow cytometric analysis.

RESULTS

Flow cytometric investigation

In Fig. 1. the histograms of light scatter and fluorescence intensity distributions of deep frozen bull semen samples, treated with NP-40 detergent and stained with PI, are presented. The fluorescence intensities were electronically gated to exclude debris and unidentifiable particles. The first peak in the fluorescence intensity distribution curve on the right side of Fig. 1. corresponds to particles having no DNA i.e. not stained with PI. The second peak with a shoulder can be assigned to sperm cells which are already dead due to NP-40 treatment (PI positive fraction). The correlation between light scatter and fluorescence intensity data of the same deep-frozen bull semen sample are shown in Fig. 2. Each point in this figure corresponds to a single cell and the coordinates of the points are proportional to the cell parameters represented by the X and Y axis. In this representation the population located in the center of the figure corresponds to PI positive particles, i.e. sperm cells. This population exhibits a remarkable fluorescence and light scatter intensity. The cell-size objects, which did not show PI uptake correspond to the population parallel to X-axis to the figure. To measure the cell number in the samples, a given number ($0.5\text{-}1 \times 10^5$) of cell-size objects were analysed and the number of PI positive objects (i.e. sperm cells) was determined. Volume of sample containing 1×10^5 of cell-size objects was determined by weighing the sample tube before and after the analysis. From these values the cell number was calculated. Distribution curves similar to those in Fig. 1. were obtained with semen samples, both fresh and equilibrated ones, applying PI staining following NP-40 treatment.

To determine the proportion and concentration of live and dead cells Fig. 3. presents the histograms of fluorescence

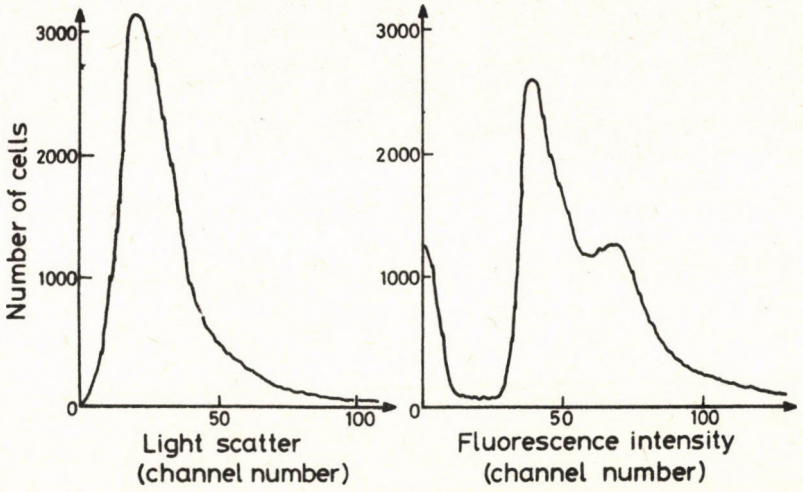

Fig. 1. Light scattering and fluorescence intensity distribution of deep-frozen semen sample treated with NP-40 and stained with PI. The cells were illuminated with a 488 nm laser beam, the fluorescent light was detected with 620 nm cut off (high pass) filter. Cells (1 x 10^6 cells/ml) were treated with NP-40 and stained with 45 μM PI at 0°C for 10 min. The histograms sum up data of 50 000 cells.

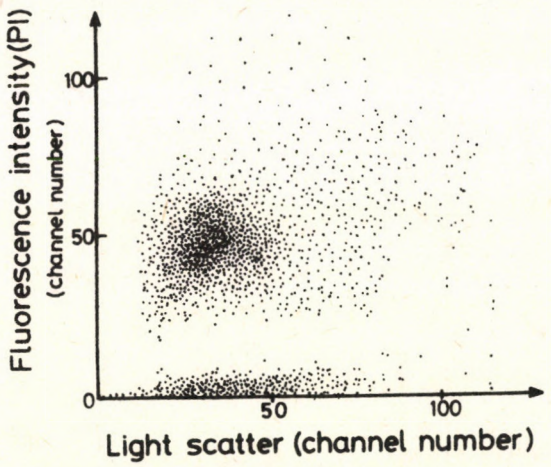

Fig. 2. Correlated representation of light scattering intensity and fluorescence intensity values of NP-40-treated and PI-stained cells. Each point in the figure corresponds to a cell. The coordinates of the chosen point are proportional to cell parameters denoted on the axes. The figure contains data of 10 000 cells. The conditions of measurement and labelling are described in the legend to Fig. 1.

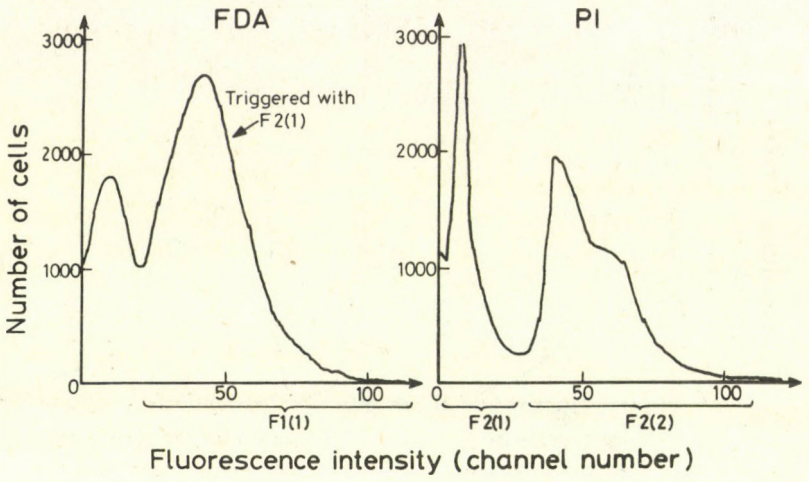

Fig. 3. Fluorescence intensity histograms of an equilibrated semen sample stained simultaneously with FDA and PI. The cells were illuminated with a 488 nm laser beam, for the detection of the green fluorescence a 540 nm interference filter, for the red fluorescence 620 nm cut off (high pass) filter was used. The staining of the sample of 1 x 10^6 cells/ml concentration was carried out with 1 µM FDA and 45 µM PI at room temperature for 8 min. The FDA distribution histogram contains the data of the objects belonging to the F2/1/ range only. The histogram summarizes data of 50 000 cells.

distribution obtained after double staining with FDA and PI of an equilibrated bull semen sample. The intensities presented on the figure are as follows: the green fluorescence intensity distribution (F1) is proportional to the fluorescein content resulting from the FDA hydrolysis of the cells, the red fluorescence intensity distribution is (F2) proportional to the PI content of the cells. The PI positive, i.e. dead cells belong to the F2/2/ range of PI distribution, the PI negative objects (live cells and other particles) are seen in the F2/1/ range. During the measurement of FDA distribution, data collection was triggered by objects having no emission i.e. with the F2/1/ range (see Fig. 3). The left-side (first) peak belongs to the FDA negative and PI negative objects which can hardly be stained with FDA or PI. The second peak of the FDA distri-

bution curve, which exhibits higher intensities (the FDA positive population in F1/1/ range, in Fig. 3) corresponds to live cells. In case of fresh semen samples the FDA positive and FDA negative populations can be clearly distinguished, however, in the frozen semen samples the two peaks are less easily discernible. When the samples were stained with FDA, fluorescein content of cells was the highest in the fresh, and lowest in the frozen semen.

Microscopic investigations

Phase contrast microscopy revealed that the overwhelming part of the particles classified by light scattering in cell-size subpopulation, proved to be sperm cells. With a frequency of a 10-15% various fragments of nearly cell size, i.e. aggregated bacteria, crystal-like contaminations, agglomerated cell fragments etc., were observed among the cells. The smaller than cell-size subpopulation contained small-size debris, detached sperm tails and sperm cells did not occur in this fraction.

Fluorescence microscopic examination of PI positive and PI negative subpopulations separated through PI staining following NP-40 treatment revealed that the PI positive subpopulation is practically free of contamination and contains exclusively sperm cells which produce uniform red fluorescence. The PI negative fraction is free of cells, it corresponds to contaminants of various size and cell fragments without red fluorescence.

The FDA positive or PI positive subpopulations of the samples labeled with double (FDA + PI) staining appeared either motile (a live) or still (dead) sperm cells, and there were no contaminating particles in this fraction as revealed by phase contrast microscopy. The FDA as well as PI negative subpopulations contained the contaminations and in a negligible amount of spermatozoa. The FDA positive cells, which show a bright green fluorescence, are motile, intact sperm cells whereas the PI positive ones are red-fluorescent, non-motile, dead sperm cells.

Determination of cell concentration in different semen samples

Applying the above described methods the number of cells in fresh, equilibrated and deep-frozen semen samples can be determined. The results are summarized in Table 1. Among the three different procedures applied the method using light scattering gave the highest, and the method summing up the live and dead cells resulted in the lowest cell number values in all samples.

Table 2 contains the correlated percentual rate of the cell concentrations of various semen samples determined by means of various procedures. In the same sample, the cell number based on light scattering measurements was usually higher by 9-14% than that of determined using all nuclei staining. Whereas the sum of live and dead cells was always less by 10-15% than the number of all nuclei stained with PI after detergent treatment.

DISCUSSION

The various methods used for flow cytometric determination of cell concentration of semen samples did not give identical results. The highest values for cell concentration can be obtained by using light scatter signal. The cell count detected through PI staining of cells after NP-40 treatment was found lower than that of based on light scattering and even lower was the value calculated as the sum of live and dead cells recorded in FDA + PI double stained samples.

The separation of sperm cell subpopulations and their subsequent microscopic examination led to the clarification of the sources of errors of particular method.

We can conclude that the particle count obtained for the sperm cell population by measuring the intensity of light scattering is usually higher than the actual cell count, and it differs from that by the extent of the contamination in the sample caused by cell-size particles. These contaminations are either partly of outward origin (grains of dust, etc.), partly come from the diluting agent (e.g. clumps of cholesterol), or

Table 1. Determination of the number of sperm cells in native, equilibrated and deep-frozen semen samples by flow cytometry, applying various staining methods

Semen sample	No. of sample	Sperm cell concentration x 10^{-6}/mm^3		
		light scattering	sum of live+dead sperm cell count**	cell nucleus staining***
native	1	1.10±0.04 (12)*	0.83±0.05 (7)	0.95±0.04 (4)
	2	1.14±0.05 (9)	0.93±0.07 (4)	1.02±0.02 (3)
	3	1.56±0.09 (2)	1.30 (1)	1.48 (1)
	4	1.93±0.02 (2)	1.42 (1)	1.82 (1)
equilibrated	1	0.91±0.02 (7)	0.70±0.02 (3)	0.78±0.03 (2)
deep-frozen	1	1.00±0.05 (6)	0.76±0.03 (3)	0.80±0.02 (3)
	2	0.89±0.02 (10)	0.67±0.03 (4)	0.73±0.10 (2)

* mean value±standard error of means (number of measurements)

** sum of sperm cells, stainable with FDA or PI

*** sperm cells treated with NP-40 and stained with PI

Table 2. Comparison of the sperm cell concentrations of bull semen samples, obtained with various methods

Type of semen sample	No. of sample	Relative sperm cell concentration		
		live+dead sperm cells sum/light scattering %	cell nucleus staining/light scattering %	live+dead sperm cells sum/cell nucleus staining %
Native	1	76.8	85.5	90.9
	2	80.0	94.0	85.0
	3	88.0	90.0	88.0
	4	73.0	95.0	78.0
	$\bar{X}$	79.5	91.1	85.3
Equilibrated	1	74.0	91.0	81.0
Deep-frozen	1	70.0	88.0	79.0
	2	75.0	84.0	89.0
	$\bar{X}$	72.5	86.0	84.0

are of endogenic origin (e.g. aggregated bacterium cells), coagulated cell fragments etc.

The sperm cell number of the samples measured with light scattering differs from the actual value by 5-15% on the average, depending on the degree of contamination.

During the treatment of the semen with NP-40, cell membranes are injured which results in the increase in the permeability of membrane. Thus, PI can easily penetrate through the membrane of cells and, having intercalated into the DNA in the nucleus, may display a bright red fluorescence. Thus, each individual sperm cell in the sample emits identical signals, which allows the unequivocal detection of the cells. This meth-

od permits the exclusion of the disturbing effect of cell-size contaminations.

The concentrations, obtained from the sum of live and dead sperm cells (FDA positive and PI positive populations) by FDA + PI double staining, is usually lower than the actual sperm cell concentration. It has been shown by microscopic examinations thet sperm cells may also occur in the subpopulation which gives neither green nor red fluorescence. These are probably those cells whose membrane has not been damaged to such an extent that PI could penetrate freely into the cell. Thus they are not really "dead", thus do not show red fluorescence, and they are unable to accumulate fluorescein and do not emit green fluorescence indicating that they are not "live". The cells in such a transitory state, i.e. the "dying cells" are not taken into consideration when cell number is determined by double staining.

The fact that cells in a transitory state do not take up PI and do not accumulate fluorescein can be explained in the following ways. The cytoplasmic membrane of the sperm cells is intact, but the intracellular esterase activity is very low. Therefore, the FDA hydrolysis within the cells is negligible, thus there is no substance to accumulate. On the other hand, due to the relative intactness of the membrane, PI still can not pass through into the cells. Another possibility may be the rate of FDA uptake is decreased as compared to that of the normal cells. Therefore these cells cannot be regarded as absolutely viable sperms.

When the staining was performed with FDA, the amount of fluorescein was found to be accumulated differently in fresh, equilibrated and deep-frozen semen samples. This fact can be explained by the influence of additive materials, such as glycerol and egg-yolk present in equilibrated and deep-frozen samples, on the degree of fluorescein accumulation. Glycerol may change the permeability of the cytoplasmic membrane both for FDA and fluorescein, or may inhibit the activity of intracellular esterases. In the egg-yolk dispersed in the freezing solution the lipophylic FDA may accumulate in, thus less FDA

is available for the cells. In order to clear up these phenomena further experiments have to be completed.

The simplest and fast, routine-like method to obtain cell number is the light scattering measurements. The cell count method based on cell nucleus staining allows the determination of cell number by light scattering, in serial examinations, if the difference caused by the proportion of cell-size particles that contaminate the semen is determined and in the subsequent measurements corrections are introduced.

Further on, the determination of the proportion of live and dead cells with simultaneous application of FDA and PI dye, as well as the 95-98% accuracy of cell concentration measurements based on PI staining after NP-40 treatment, permit:

- considerably more accurate calibration of the routine laboratory instruments for sperm cell count;
- measurements of the proportion of live and dead spermatozoa and minute change in their proportion;
- clearing up the objective relationship between the sperm cell count and the proportion of live cells of the insemination dose and fertility;
- elaboration of working principles of equipment for the determination of the total sperm count and the ratio of live and dead sperm cells.

This equipment can be applied on routine laboratory level to measure the parameters characterizing the quality of the semen with adequate accuracy.

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INHIBITORY EFFECT OF A 23187 ON PROTEIN SYNTHESIS IN ISOLATED MURINE HEPATOCYTES

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SUMMARY

The effect of the Ca ionophore A 23187 on amino acid incorporation into protein, on gluconeogenesis and on synthesis of some eicosanoids was investigated in isolated mouse hepatocytes (i) in the presence of 2.5 mM Ca^{2+}, (ii) in the absence of Ca^{2+} and in the presence of 0.2 mM EGTA, (iii) after calcium depletion. A 23187 inhibited amino acid incorporation into protein both in the presence and in the absence of Ca^{2+} in a concentration dependent manner, whereas in the absence of Ca^{2+} the known stimulating effects of A 23187 on eicosanoid synthesis could not be detected. In hepatocytes prepared from 24 h starved mice A 23187 increased the rate of gluconeogenesis and similarly to eicosanoid synthesis this stimulation in the absence of Ca^{2+} could not be observed. On the other hand, after calcium depletion the inhibition of protein synthesis by A 23187 was markedly moderated. It is concluded that the effect of A 23187 on protein synthesis is also related to its ionophoretic effect but calcium sensitivity in this effect is smaller than in gluconeogenesis or eicosanoid synthesis.

INTRODUCTION

Ca^{2+} is known to mediate various effects of several hormones and neurotransmitters (1, 2). A 23187 is a Ca^{2+} ionophore antibiotic and has proved to be a very useful experimental tool to model calcium dependent processes in the cells. We have pre-

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viously found that epinephrine inhibits protein synthesis in a Ca^{2+} dependent way in isolated mouse hepatocytes (3). In agreement with our observations Grinde (4) has described that A 23187 inhibits protein synthesis in rat hepatocytes. However, it has been also reported that A 23187 was inhibitory on protein synthesis without addition of extracellular calcium and furthermore Ca^{2+} moderated this inhibition. These findings were not in accordance with the various known effects of A 23187 on several processes of intermediary metabolism, which have been related to its Ca^{2+} ionophoretic effect (4-13).

In isolated hepatocytes A 23187 similarly to different chemicals (D-galactosamine, ethanol etc., frequently called "membrane target" agents) causes also necrosis of the cells in the course of a 24 h incubation, but in the absence of extracellular Ca^{2+} necrosis is not observed (14, 15). However, in case of galactosamine or ethanol it has been shown that their inhibitory effect on protein synthesis has been independent of extracellular calcium (16).

These observations and the mentioned results of Grinde (4) raised the possibility that the inhibitory effect of A 23187 on protein synthesis is independent of its Ca^{2+} ionophoretic effect. To investigate this problem experiments were undertaken to examine the effect of A 23187 on protein synthesis in the presence and in the absence of calcium or in calcium depleted hepatocytes. In order to investigate how A 23187 affected other processes under our experimental conditions the effects of A 23187 on gluconeogenesis and on eicosanoid synthesis were also studied. The inhibitory effect of A 23187 was manifested both in the presence and absence of Ca^{2+} but only a minor inhibition was found in calcium depleted cells.

MATERIALS AND METHODS

Isolated mouse hepatocytes were prepared from CFLP mice fed ad libitum by the collagenase perfusion method as detailed previously (17). The isolated cells (5×10^6/ml) were incubated in Krebs-Henseleit bicarbonate buffer containing 2.5 mM Ca^{2+}, 8.5 mM glucose, 5 mM lactate, all amino acids necessary for protein synthesis (1 mM each except valine) and 1% albumin.

In a series of experiments calcium was omitted from the incubation medium and as the calcium content of this "Ca^{2+} free" medium was lower than 10^{-4} M 0.2 mM EGTA was added as described previously (3). Another series of experiments was performed in calcium depleted hepatocytes. Calcium depletion was carried out using 1 mM EGTA for 20 min as described by Chan et Exton (18). The cells were stirred by constant bubbling with O_2:CO_2 (95:5 v/v) at 37°C.

Viability of the cells checked by the trypan blue exclusion test was about 90-95%.

Protein synthesis in isolated hepatocytes was investigated by determination of ^{14}C-valine incorporation (65 TBq/mole, 18.5 KBq/ml) into the hot trichloroacetic acid insoluble material of the cells as described earlier (16).

Gluconeogenesis in isolated hepatocytes prepared from 24 h starved mice was measured as described previously (19). Glucose production was measured by Bergmeyer (20).

The contents of TXB_2, 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were measured by radioimmunoassay using RIA kits of the Institute of Isotopes, Budapest, Hungary.

DNA content of the cells was measured according to Burton (21).

Materials: L-[U-^{14}C]-Valine was purchased from UVVVR, Prague, Czechoslovakia, A 23187 from Calbiochem, La Jolla, Ca, USA, Collagenase (type IV) from Sigma, St. Louis, Mo, USA. All reagents were of analytical grade.

RESULTS

Isolated hepatocytes were incubated (i) under standard incubation conditions in the presence of 2.5 mM Ca^{2+}; (ii) without Ca^{2+} and in the presence of 0.2 mM EGTA; (iii) after calcium depletion and the effect of A 23187 on amino acid incorporation into proteins was investigated. As shown in Table 1 the absence of extracellular Ca^{2+} and the parallel addition of 0.2 mM EGTA did not cause a significant change in the rate of amino acid incorporation in the course of a 30 min incubation. The addition of A 23187 inhibited amino acid incorporation into proteins. This inhibition was dependent on concentration of A 23187 and also on time (Fig. 1). Amino acid incorporation into proteins was decreased by about 40% in the presence of 10^{-5} M A 23187 (Table 1), but this inhibition was manifested only after at least 10 min incubation (Fig 1). The lack of calcium in the incubation medium did not alter the inhibitory effect of A 23187 in time course or in extent. However if the cells were calcium depleted the inhibition by A 23187 was markedly moderated (Table 1).

Table 1. Effect of extracellular Ca^{2+} concentration on A 23187 caused decrease of amino acid incorporation into protein in isolated hepatocytes

Isolated mouse hepatocytes were incubated in the presence and in the absence of Ca^{2+} or after Ca^{2+} depletion for 30 min. In the absence of Ca^{2+} 0.2 mM EGTA was added. The incorporation of ^{14}C-Valine into proteins was measured. Mean± S.E.M. (n = number of observations made in different experiments). different from control at $^{*}p < 0.01$ and $^{**}p < 0.1$ level

Addition	^{14}C-Valine incorporation into protein (%)		
	2.5 mM Ca^{2+}	Ca^{2+} "free"	Ca^{2+} depleted
none	100	91.0±7.2 (10)	106.2±18.0 (4)
10^{-5} A 23187	58.5±9.9 (7)*	43.5±4.5 (7)*	86.1± 8.1 (4)**

The calcium dependence of other effects of A 23187 was investigated under our experimental conditions. In isolated hepatocytes prepared from 24 h starved mice gluconeogenesis was increased when 10^{-5} M A 23187 was added (Table 2), but this stimulation was diminished in cases when Ca^{2+} was omitted from the incubation medium (and 0.2 mM EGTA was added). Similar results were obtained when the stimulation of eicosanoid synthesis by A 23187 was investigated. As expected 10^{-5} M A 23187 stimulated the synthesis of thromboxane by 404±64%, of prostacycline by 193±14% and also the production of $PGF_{2\alpha}$ by 253±10% (mean±S.E.M. n = 4) in a 5 min incubation. However, in the absence of Ca^{2+} no stimulation could be detected.

DISCUSSION

In this paper the Ca^{2+} dependence of the inhibitory effects of A 23187 on protein synthesis is demonstrated. In an accordance with the results of Grinde (4) A 23187 causes an inhibition of protein synthesis in murine hepatocytes, however Ca^{2+} does not moderate this inhibition (Fig. 1). Thus, contrary to other "membrane target" agents as galactosamine or ethanol

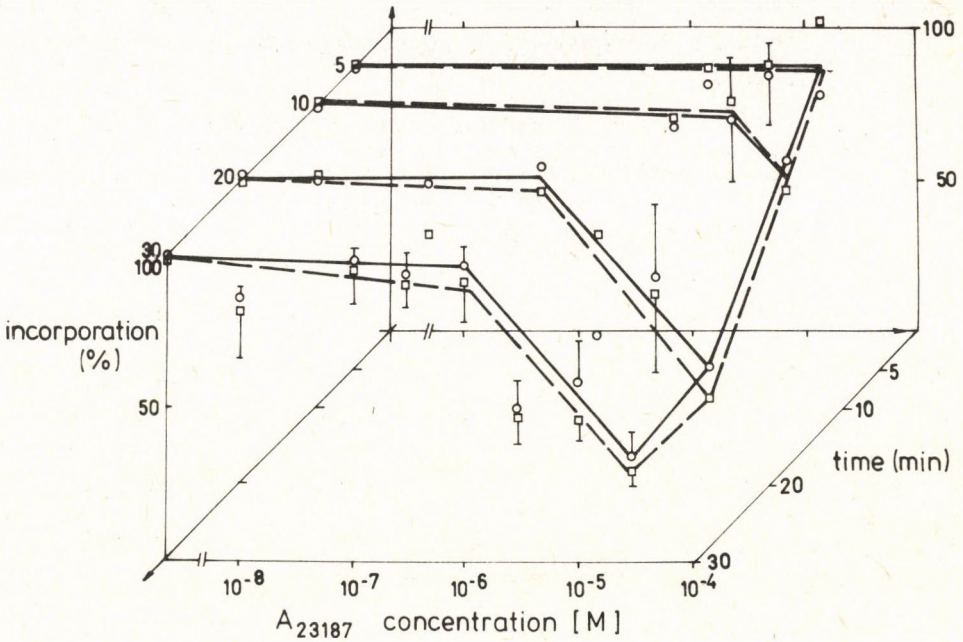

Fig. 1. Effect of A 23187 on amino acid incorporation into protein in isolated hepatocytes. The effect of A 23187 on ^{14}C-Valine incorporation into protein was investigated in the presence of 2.5 mM Ca^{2+} (o) or in the absence of that and in the presence of 0.2 mM EGTA (□). In the absence of Ca^{2+} and in the presence of 0.2 mM EGTA the ^{14}C-Valine incorporation was 130.4±9.4% (3) at 5 min, 115.9±11.1% (3) at 10 min, 103.2±19.3% (3) at 20 min and 91.1±7.2% (10) at 30 min of the control (measured in the presence of 2.5 mM Ca^{2+}) value. Vertical bars indicate S.E.M. (n = 3-7).

(14, 15), which besides their membrane effects have a distinct inhibitory effect on protein synthesis (16), the A 23187 provoked inhibition is due to its Ca^{2+} ionophoretic effect. On the other hand, the sensitivity to extracellular Ca^{2+} of this inhibitory effect by A 23187 was smaller; the stimulating effects on gluconeogenesis and on eicosanoid synthesis have already been stopped under "Ca^{2+}free" conditions, while it was still inhibitory on amino acid incorporation on protein synthesis (Tables 1, 2). It can be suggested that the mobilized intra-

Table 2. Effect of extracellular Ca^{2+} concentration on A 23187 induced increase of gluconeogenesis in isolated hepatocytes

Isolated hepatocytes were prepared from 24 h starved mice and were incubated with 10 mM lactate in the presence and in the absence of Ca^{2+} or after calcium depletion for 30 min. In the absence of Ca^{2+} 0.2 mM EGTA was added. The glucose production was measured. Mean ± S.E.M. (n = number of observations made in different experiments).

Addition	Glucose production (nmole/g liver/min)		
	2.5 mM Ca^{2+}	Ca^{2+} "free"	Ca^{2+} depleted
none	157±24 (4)	176±65 (4)	107±19 (3)
10^{-5} M A 23187	247±42 (4)	145±43 (4)	83±21 (3)

cellular Ca^{2+} by A 23187 (6) is sufficient to this inhibition, while after calcium depletion the required Ca^{2+} is not available within the cells.

The Ca^{2+} dependent inhibitory effect of A 23187 is in accordance with the Ca^{2+} dependent inhibition by epinephrine on protein synthesis (3) and supports the assumption that a Ca^{2+} dependent regulation of protein synthesis exists. A cAMP dependent (and also cAMP independent) control of translation has been described first in heme controlled protein synthesis of rabbit reticulocytes (22, 23). The hemin controlled inhibition of 5-amino-levulinate synthase induction in isolated hepatocytes has been described recently (24). Ca^{2+} and cAMP mediated regulations are usually parallel in isolated hepatocytes (25, 26). Dibutyryl cAMP has been also shown to inhibit protein synthesis in isolated hepatocytes (3, 27). This calcium dependent inhibitory effect of A 23187 on protein synthesis is in accordance with the other known metabolic effects, which model the effects of various effectors acting via Ca^{2+} mediated receptors. A 23187 is also known to stimulate glucose production (5), to increase

the amino acid transport into hepatocytes (9) and at the same time it inhibits the diversion of gluconeogenic intermediates for NADPH generation to supply mixed function oxidation (28). Therefore, it is tempting to suppose that the Ca^{2+} mediated inhibition of protein synthesis shown with epinephrine (3) and A 23187 might be a part of regulation, which serves to support the substrate supply of gluconeogenesis.

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ENZYMATIC HYDROLYSIS OF ELECTRICALLY CHARGED SUBSTRATES

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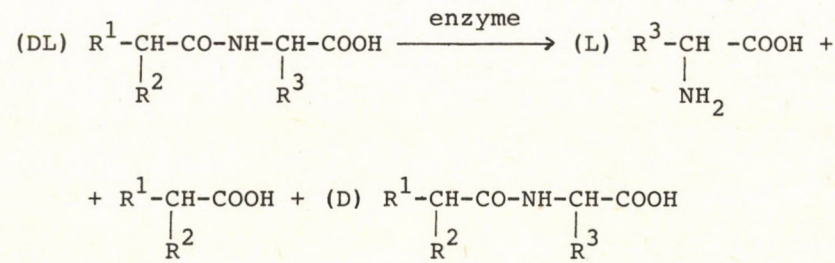
SUMMARY

A study has been made on the hydrolytic action of porcine kidney aminoacylase I (EC 3.5.1.14.) upon electrically charged substrates containing ionic groups both in the acyl moiety and in the C-terminal amino acids. In all cases a decrease in reactivity was found. An attempt has been made to elucidate the nature of this action.

INTRODUCTION

Aminoacylase I is one of the most important enzymes employed in organic chemical industry. It catalyzes the hydrolysis of acyl amino acids to the following scheme:



Earlier studies on the mechanism and the sterospecificity of the enzyme in the hydrolytic reaction of acyl amino acids and dipeptides (Fodor et al., 1949a; 1949b; Birnbaum et al., 1953; Fu et al., 1953; Fones et al., 1953; Fones et al., 1954; Mounter et al., 1958; Tsuchray et al., 1979; Shviadas et al., 1980; Ötvös et al., 1971; Ötvös et al., 1977; Moravcsik et al.,

1977) showed that the stereospecificity of these reactions is of kinetic nature, which has been explained quantitatively by the fixed conformation of substrates in the enzyme-substrate complex (Ötvös et al., 1975). The different electrically charged groups in the enzyme and in substrates play the most important role in binding the substrate molecule, which determines the specificity and stereospecificity of the aminoacylase toward the corresponding substrate. Therefore, we made a systematic study of the effect of different ionic groups on the enzymatic reaction. In this paper the hydrolysis of different substrates containing negatively and positively charged groups both in C- and N-terminal moietis has been described.

MATERIALS AND METHODS

The following compounds have been synthetized:

I. $R^1-(CH_2)_n-CH(NH-CO-CH_3)-COOH$

$R^1 = CH_3$; COOH; $COOR^2$; $CONH_2$

$R^2 = CH_3$; C_2H_5

n = 0; 1; 2

II. $R^1-CH(NH-CO-CH(R^3)-CH_2-R^2)-COOH$

$R^1 = CH_3$; n-C_3H_7

$R^2 = CH_3$; C_2H_5; NH_2; CH_2-NH_2; COOH; CH_2-COOH

$R^3 = H$; NH_2

Most of these compounds have been reported earlier. The acylated amino acids were prepared by the usual procedures from the amino acids (Greenstein and Winitz, 1961; Breslow et al., 1944; Birnbaum et al., 1952; Marshall et al., 1956; Valentekovic et al., 1976). The peptides were synthesized either by the phosphazo method (Goldschmidt et al., 1953) or by the reaction of carbobenzoxy derivatives of amino acids with amino acid ethyl or methyl ester in organic solvent mixture, using iso-valeryl chloride or dicyclohexyl carbodiimide as condensating reagents (Grassman et al., 1958; Fu et al., 1954).

Porcine kidney aminoacylase I was purchased from the Koch-Light Laboratories LTD. The specific activity of the enzyme was determined according to Bruns and Schultze (Bruns and Schultze, 1962). All materials were purified to constant melting points and optical rotation. Purity was checked by elementary analysis, IR and NMR spectrometry as well as thin layer chromatography.

Kinetic procedure: enzymatic hydrolysis was carried out in phosphate buffer pH 7.15, at 37°C, and followed in a PVE-UNICAM spectrophotometer. $K_{m(app)}$ and V_{max} values were determined from Lineweaver-Burk plots, and k_{cat} values were calculated from the relationship $k_{cat} = V_{max}/E$. Enzyme concentration ranged between 4×10^{-8} M and 4.5×10^{-7} M based on 86 000 molecular weight (Kördel et al., 1976), and substrate concentrations were between 3×10^{-3} M and 3×10^{-2} M.

RESULTS AND DISCUSSION

Table 1 contains kinetic data observed for substrates of general formula I.

In the case of acyl aspartic acid instead of $K_{m(app)}$ the K_I value was determined, which also characterizes the binding force between the enzyme and the substrate.

Comparison of the $K_{m(app)}$ constants showed that ionic groups in substrates decrease the binding capacity, also verified by the enhanced $K_{m(app)}$ values. This is in accordance with the diminished $K_{m(app)}$ constants when ionic groups are removed from substrate molecules: i.e., the carboxyl group is converted either into an esther or an amide group. The change in $K_{m(app)}$ values of this type may be interpreted by the fact that in the presence of an ionic group in the hydrophobic chain of the substrates, the hydrophobic binding side of amino-acylase I is unable to bind the large, hydrated, electrically charged group. This gives an explanation for the decrease in binding strength. Naturally, the removal of ionic groups from the substrate (when preparing an ester on an amide group from a carboxyl group) partly restores the hydrophobic nature of a side chain, and thus improves the binding capacity - especially at a distance from the reaction centre (n = 2). In the case of α-chymotrypsin similar effects have been observed: the introduction of a positively or negatively charged ionic group into a substrate reduces reactivity greatly.

Table 1. Kinetic constants for substrates of general formula I

C O M P O U N D (N-acetyl-)	$K_{m(app)}$ mM	k_{cat} s^{-1}	$k_{cat}/K_{m(app)}$ $mM^{-1}s^{-1}$
$CH_3-CH(NH-)-COOH$	22.1	112.8	5.10
$HOOC-CH(NH-)-COOH$	138.0	2.2	0.02
$C_2H_5OOC-CH(NH-)-COOH$	136.0	41.8	0.31
$CH_3-CH_2-CH(NH-)-COOH$	6.9	155.0	22.50
$HOOC-CH_2-CH(NH-)-COOH$	K_i=39	-	-
$CH_3OOC-CH_2-CH(NH-)-COOH$	14.0	3.3	0.20
$H_2N-CO-CH_2-CH(NH-)-COOH$	12.7	2.6	0.20
$CH_3-(CH_2)_2-CH(NH-)-COOH$	4.8	160.0	33.30
$HOOC-CH_2-CH_2-CH(NH-)-COOH$	78.0	157.4	2.02
$CH_3OOC-CH_2-CH_2-CH(NH-)-COOH$	21.2	72.2	3.40
$H_2N-CO-CH_2-CH_2-CH(NH-)-COOH$	23.5	117.9	5.0

The observation of k_{cat} values clearly shows that electrically charged groups in substrates, especially near the reaction centre (n = 0, 1), greatly decrease reaction velocity. The elimination of the ionic group in a substrate increases reaction rate. In the case of glutamic acid (n = 2) the presence or absence of an ionic group far from reaction centre does not especially effect the k_{cat} value. This observation may be explained by polar effect caused by ionic groups. As pointed out earlier, in the enzymatic reaction catalyzed by aminoacylase I polar effects have an important role in reactivity.

According to the Taft-Ingold equation (Taft, 1965), electron with drawing groups enhance and electron repulsing groups slow down the velocity of the enzymatic reaction. The highly electron repulsing carboxyl group decreases the reaction rate. This effect is essential only if the carboxyl group is near to the reaction centre. The polar effect diminishes along CH_2 groups, and thus in the case of acyl glutamic acid there is no substantial difference between hydrolytic rates of ionic or non-ionic substrates.

Table 2 depicts the kinetic constants of substrates containing ionic groups in the acyl moiety.

Observing the $K_{m(app)}$ values, we may conclude that the presence of ionic groups decreases the binding strength, irrespective of the charge. Replacements of a methyl group at the end of the side chain by a carboxyl or an amino group cause a similar increase in the $K_{m(app)}$ value.

The -amino group has an interesting effect:

a. it also reduces the binding capacity, although much less than in substrates having an amino group distant from the reaction centre;

b. an -amino group moderates the influence of another amino group distant from the reaction centre, i.e. the $K_{m(app)}$ values hardly change.

Positively or negatively charged groups - either in a near or in a distant position - have comparable effects on k_{cat} constants, reducing catalytic activity similarly.

If the same molecule has two charged groups, owing to their common effect on the k_{cat} values, this substrate is hy-

Table 2. Kinetic constants of substrates containing ionic groups in the acyl moiety

C O M P O U N D (-L-Ala)	$K_{m(app)}$ mM	k_{cat} s^{-1}	$k_{cat}/K_{m(app)}$ $mM^{-1}s^{-1}$
$CH_3-CH_2-CH_2-CO-$	1.8	52.8	29.30
$HOOC-CH_2-CH_2-CO-$	140.0	6.8	0.05
$CH_3-CH_2-CH_2-CH_2-CO-$	1.0	40.0	40.00
$HOOC-CH_2-CH_2-CH_2-CO-$	200.0	4.5	0.02
(L) $CH_3-CH_2-CH_2-CH(NH_2)-CO-$	3.4	10.6	3.10
(L) $H_2N-CO-CH_2-CH_2-CH(NH_2)-CO-$	54.0	0.7	0.013
(L) $HOOC-CH_2-CH_2-CH(NH_2)-CO-$	6.1	0.6	0.10
(D) $HOOC-CH_2-CH_2-CH(NH_2)-CO-$	K_i=4.8	-	-
(L) $HOOC-CH_2-CH(NH_2)-CO-$	16.4	0.72	0.04
(-L-Nva)			
$CH_3-CH_2-CH_2-CH_2-CO-$	0.4	160.00	400.00
$HOOC-CH_2-CH_2-CH_2-CO-$	120.0	21.00	0.18
$CH_3-CH_2-CH_2-CO-$	0.7	180.00	260.00
$H_2N-CH_2-CH_2-CH_2-CO-$	200.0	18.00	0.09
(L) $CH_3-CH_2-CH_2-CH(NH_2)-CO-$	2.7	102.00	38.00
(L) $HOOC-CH_2-CH_2-CH(NH_2)-CO-$	4.0	1.90	0.50
(L) $H_2N-(CH_2)_4-CH(NH_2)-CO-$	4.7	0.09	0.02

drolyzed at a very low rate, irrespective of the charge of the groups. This independence of the character of ionic groups indicates that this effect is of non-polar character. The decrease of the binding capacity is probably due to a steric hindrance of the hydrate shell around charged groups or to an inadequate linkage in the E-S complex, which leads to a change in conformation. The anomalous behaviour of acyl aspartic acid requires further investigation.

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IN VITRO EFFECT OF EMETINE AND CHLOROQUINE ON THE MACROMOLECULAR BIOSYNTHESIS OF MURINE THYMUS CELLS

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SUMMARY

The in vitro effect of antiparasitic agents, emetine and chloroquine on the DNA, RNA and protein synthesis was studied in isolated murine thymocytes. Cytotoxic effect was observed at 10^{-4} and 10^{-3} M emetine concentrations causing 50% and 70% cell death, respectively. The toxic effect of emetine could be prevented when emetine was removed within 10 min of treatment. Inhibitory concentrations for DNA, RNA and protein biosynthesis were 10^{-5}, 5×10^{-5} and 10^{-8} M, respectively. The number of living cells decreased by 30 and 50% at 10^{-4} and 10^{-3} M chloroquine concentrations, respectively. Gradually decreasing rate of DNA synthesis was measured at increasing concentration of chloroquine between 10^{-8} and 10^{-3} while RNA and protein synthesis were effected at 5×10^{-5} M concentration. These results indicate that protein biosynthesis is primarily affected by in vitro emetine and chloroquine treatment of murine thymocytes.

INTRODUCTION

Ipecac alcaloids including emetine have been used for medical purposes for over 300 years. The therapeutic spectrum of emetine was further broadened in this century by using it as amebicid drug (Vedder, 1912) and against granulomas (Grollman, 1965). Emetine is a potent inhibitor of in vitro protein and DNA synthesis of mammalian cells (Grollman, 1966; Grollman, 1968). The inhibition of RNA synthesis by emetine was also reported (Perry and Kelly, 1968; Gilead and Becker, 1971; Farkas

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et al., 1974). The immune response and phagocytic processes are at least temporarily suspended by emetine (Antoni et al., 1986; Csuka and Antoni, 1984). Emetine inhibits in vivo biosynthesis of macromolecules in lymphocytes (Antoni et al., in press).

Chloroquine, a lysosomotropic drug is extensively used in the treatment and prophylaxis of malaria, against amebiasis, rheumatoid arthritis, lupus erythematosus (Pomerantz, Smith and Malak, 1963; Rollo, 1970; Zwaifleer, 1971). The anti-inflammatory property of chloroquine is believed to be due to its ability to stabilize lysosomal membranes (Weissmann et al., 1963), the suppress the lymphocyte responsiveness (Hurwitz and Hirshhorn, 1965) and the protein synthesis (Forbes and Smith, 1967) and the inhibition the chemotaxis in leucocytes (Ward, 1966). These observations suggest that chloroquine may also act in vivo on immuncompetent cells (Prakash et al., 1980).

The in vivo immunosuppressive potential of antiparazitic agents, such as emetine and chloroquine has to be supported by in vitro measurements. However, a detaliled analysis of the in vitro effect of these drugs on lymphoid cells is missing. Thus we have carried out experiments using the central immune organ, thymus. This paper describes that in thymocytes protein biosynthesis is the process which is primarily affected by emetine and by higher concentrations of chloroquine. The in vitro synthesis of nucleic acids is also influenced by emetine but not by chloroquine.

MATERIALS AND METHODS

Preparation of cell suspension: Male CFLP mice, 8-10 weeks of age were used. Cells were gently removed from thymus, after washing them three times with Hank's solution and resuspended in the same medium. Tne suspension contained usually 10^7 cells per ml.

Cytotoxicity of emetine

Incubations were carried out in 1.0 ml of Hank's solution at 37°C. The reaction mixture contained cells (10^7) and various inhibitors at concentrations indicated. The living and dead cells were counted in percents of living cells versus untreated cells. Counting was based on trypan blue dye uptake and exclusion.

DNA synthesis

The reaction mixture (1.0 ml) contained 10^7 cells, 37 kBq Methyl-^{3}H thymidine (925 GBq/mmole) and the inhibitors. It was incubated at 37°C for 60 min unless otherwise indicated. The reaction was terminated by the addition of 1.0 could 1 M perchloric acid (PCA). The precipitate was washed three times with cold 0.5 M PCA and the samples were hydrolyzed with 1.0 ml 0.5 M PCA at 90°C for 30 min. Aliquots (0.2 ml) were added to 5 ml of toluene based scintillation coctail and the radioactivity was measured in Beckman LS 335 liquid scintillation spectrometer.

Determination of DNA

The DNA was measured according to the method of Burton (4) using an ELKO III photometer.

RNA synthesis

For the determination of RNA synthesis the reaction mixture (1.0 ml) contained 165 kBq uridine-5-^{3}H (914 GBq/mmole), 10^7 cells and inhibitors. The reaction was carried out at 37°C for 60 min if not indicated otherwise. Reaction was stopped by the addition of an equal volume of 1 M perchloric acid. The radioactivity of the PCA insoluble fraction was measured. RNA content was determined by the orcinol reaction method (Ceriotti, 1955).

Protein synthesis

The incorporation of ^{14}C -DL-valine (7.46 MBq/mmole) was carried out in 1.0 ml Hank's solution. The reaction mixture contained 37 kBq ^{14}C-valine, 10^7 cells and various inhibitors. At the end of the incubation lasting for 60 min normally, an equal volume of 1 M PCA was added to the incubation mixture. Acid precipitable material was washed 3-times with cold 0.5 M PCA. Samples were dissolved in 1.0 ml 0.5 M NaOH for 30 min. Radioactivities of samples were measured. The protein content was determined by the method of Lowry (1951).

Reversibility of inhibitory effect of emetine

The reversibility was tested by exposing cells to a 30 min pulse of emetine at 37°C in Hank's solution. The cells were washed twice with Hank's solution and resuspended in the same solution. The incubation continued for another 30 min in the absence of emetine.

RESULTS

Cytotoxic effect of emetine on thymic cells

Early clinical investigations of emetine administration revealed its temporary cardiotoxicity without permanent cardiovascular changes especially at total doses as high as 20-25 mg/kg (Dack, Moloshok, 1947; Klatskin, Friedman, 1948). These observations served as a basis for the test of the toxic effect of emetine in mouse thymocytes. The number of living cells is

dependent on the emetine concentration (Fig. 1a) and on the time of its presence in the medium (Fig. 1b). About 50% of cells died at 10^{-4} M emetine concentration at 37°C within 60 min and 70% at 10^{-3} M emetine concentration under the same conditions. The cytotoxicity was reversible if emetine was removed within 10 min of treatment and replaced by emetine-free medium.

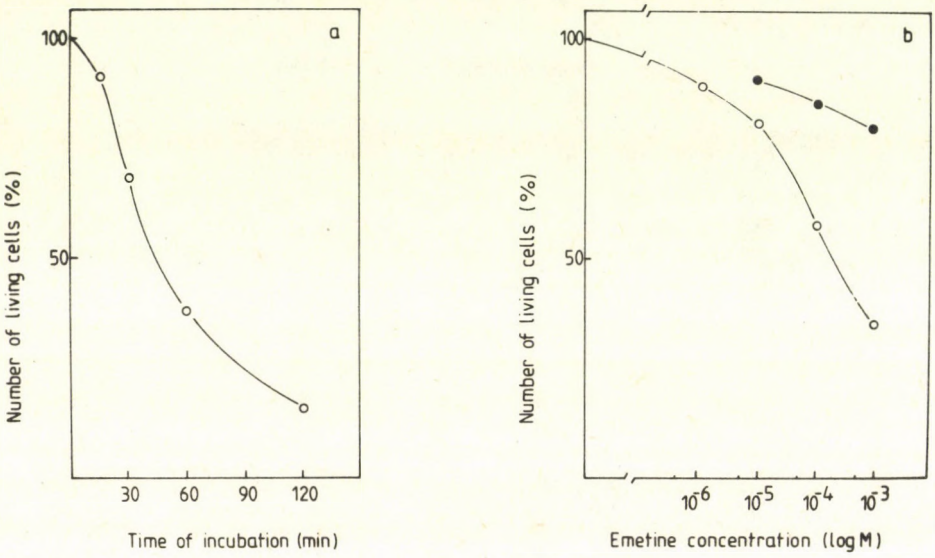
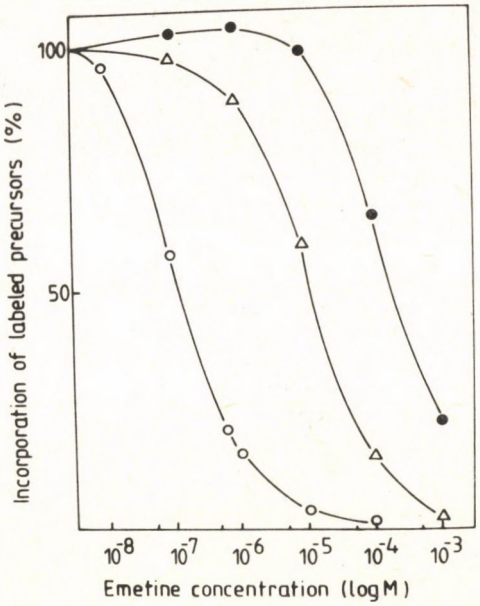


Fig. 1. Cytotoxic effect of emetine. Cell number as a function of a. time of incubation in the presence of 10^{-3} M emetine and b. emetine concentration. Incubation was at 37°C for 60 min (o), or for 10 min followed by the removal of emetine and incubated for further 50 min in the absence of emetine (●). The number of cells were expressed in percent of the control cell population.

Effect of emetine concentration on macromolecular biosynthesis

The in vitro rates of DNA, RNA and protein synthesis were compared by measuring the incorporation of radioactive precursors as a function of emetine concentration (Fig. 2). Protein synthesis proved to be the most sensitive process inhibited at low concentrations while DNA synthesis was inhibited at higher concentrations of emetine. The rate of RNA synthesis was reduced only at relatively high concentrations of emetine. The



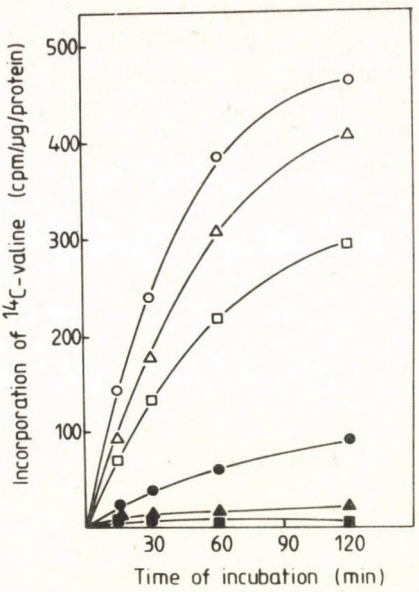


Fig. 2. (left) Effect of emetine on the biosynthesis of macromolecules. Incorporation of labeled precursors is described in the "Methods". Rate of DNA (△), RNA (●) and protein (o) biosynthesis is expressed in percents of the incorporation in untreated cells.

Fig. 3. (right) Effect of emetine on the kinetics of protein biosynthesis. Incorporation of ^{14}C-valin was measured at different intervals at emetine concentrations as follows: 10^{-4} (■), 10^{-5} (▲), 10^{-6} (●), 10^{-7} (□), 10^{-8} M (△) and in the absence of it (o).

kinetic analysis of inhibition of macromolecular biosynthesis reveals that emetine concentration which prevents protein synthesis optimal is in the order of magnitude of 10^{-7} to 10^{-6} M (Fig. 3), 10^{-5} to 10^{-4} M for DNA (Fig. 4) and 10^{-4}-10^{-3} M for RNA synthesis (Fig. 5).

Cytotoxicity of chloroquine

The number of living cells was tested at different concentrations of chloroquine. Fig. 6 shows that significant changes compared to untreated cells occur at 10^{-4} M concentration of chloroquine. The time course of chloroquine treatment at 10^{-3} M indicated that at least 60 min incubation period was

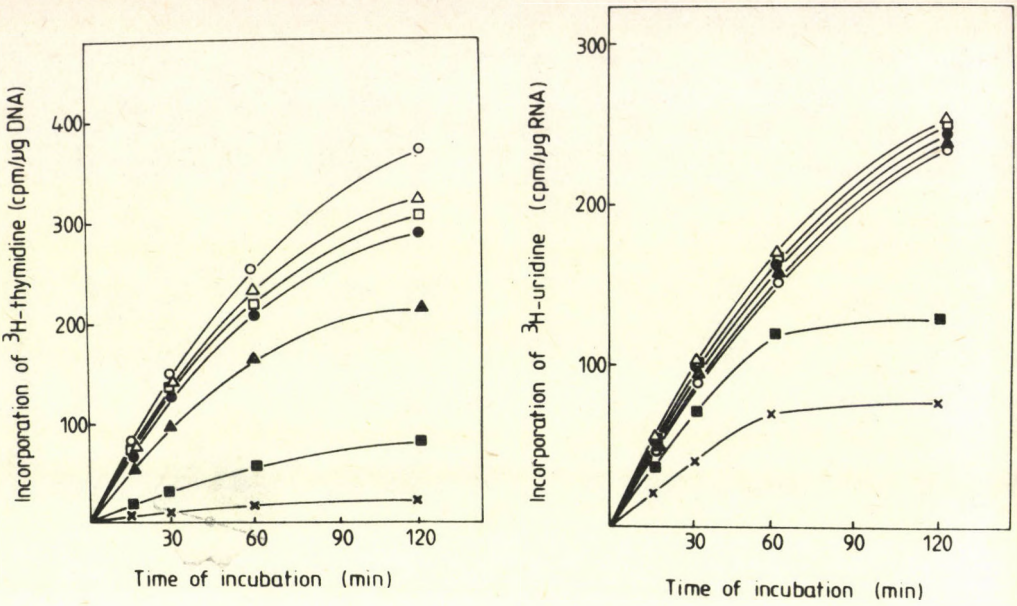


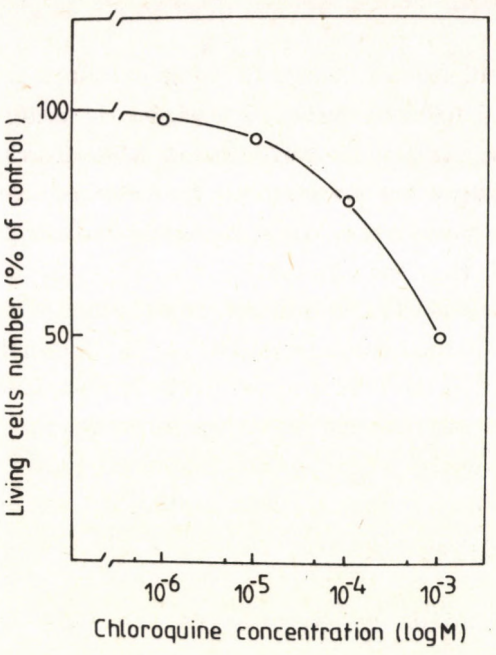
Fig. 4. (left) Effect of emetine on the kinetics of DNA synthesis. Incorporation of ^{3}H-thymidine in the presence of 10^{-3} (x), 10^{-4} (■), 10^{-5} (▲), 10^{-6} (●), 10^{-7} (□) and 10^{-8} M (△) emetine concentration and in the absence of the drug (o).

Fig. 5. (right) Effect of emetine treatment on the protein synthesis. Incorporation of ^{14}C-valine in the presence of different concentrations of emetine. Symbols are the same as in Fig. 4.

necessary to cause 50% of cells to die. The cytotoxic effect was reversible when chloroquine was removed within 30 min of treatment (results not shown).

Effect of chloroquine on the macromolecular biosynthesis

The rates of DNA, RNA and protein synthesis were measured as a function of chloroquine concentration (Fig. 7). Gradual decrease of DNA synthesis at increasing concentrations of the drug was contrasted with the rapid drop of protein synthesis at higher concentrations than 10^{-5} M. RNA synthesis was moderately increased at low (10^{-8}-10^{-6} M) concentrations and drastically reduced at higher (10^{-4} M) concentrations of chloroquine.



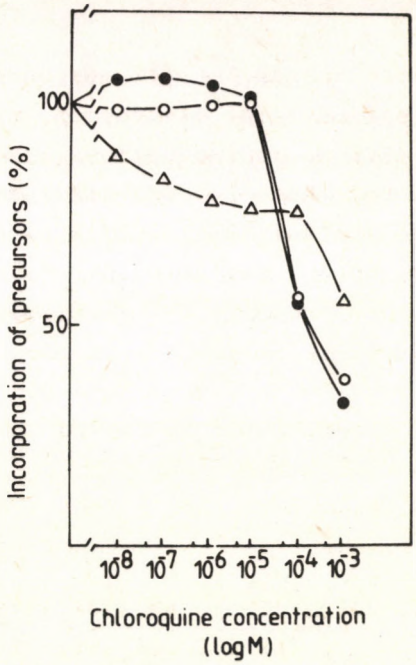


Fig. 6. (left) Number of living cells as a function of chloroquine concentration. 10^7 cells were incubated at various concentrations of chloroquine at 37°C for 60 min. Cells were counted and expressed as the percent of the control cell population.

Fig. 7. (right) Effect of chloroquine on the macromolecular biosynthesis. Incorporations of precursors are expressed as percents of the control. Synthesis of DNA (Δ), RNA (●) and protein (o). Experimental details are described in the "Methods" section.

DISCUSSION

We reported earlier the in vitro effect of emetine on protein and RNA synthesis in human tonsillar lymphocytes (Farkas et al., 1974) and the in vivo effects of emetine on the production of specific antibodies in mice (Csuka and Antoni, 1984). Morpho-functional changes in mouse thymus upon emetine treatment were also observed (Antoni et al., in press). Studies of in vitro effects of emetine and chloroquine on phagocytic processes (Antoni et al., 1986) broadaned the scale of investigations and led to the comparison of these antiparasitic drugs with respect to their in vitro inhibition of macromolecular biosynthesis.

Present data confirm earlier findings in other cells and suggest that protein synthesis is the primary target of emetine treatment in mouse thymocytes, as well. The common blocking effect of emetine and chloroquine on protein synthesis is contrasted with a relatively low reduction of synthetic rate of nucleic acids by chloroquine. This difference is not surprising since emetine and chloroquine are different in chemical nature, emetine consisting of two isoquinoline rings while chloroquine contains one quinoline ring. The inhibitory effect of emetine in eukaryotes is due to its interference with an anabolic process, namely with ribosome translocation along mRNA (Gupta and Siminovitch, 1977), while chloroquine, a lysosomotropic amine, inhibits protein catabolism by increasing lysosomal pH (Unanue, 1984), thus is not directly related to protein synthesis.

The synthesis of nucleic acids by emetine is significantly depressed. This fact might be related to the suppression of protein factors involved in nucleic acids biosynthesis (Black et al., 1967). In vitro data are consistent with our recent in vivo measurements showing a time dependent divergence of DNA, RNA and protein synthesis in mouse thymocytes (Antoni et al., in press).

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EFFECT OF THE pKM101 PLASMID ON THE REPAIR OF SINGLE-STRAND BREAKS IN DNA INDUCED BY IONIZING IRRADIATION IN ESCHERICHIA COLI

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SUMMARY

The effect of pKM101 plasmid on repair of single-strand breaks in DNA induced by ^{60}Co-gamma irradiation in E. coli K12 AB1157 (wild type) and in its recA$^-$ and recB$^-$ mutant cells was studied by alkaline sucrose gradient sedimentation method. For quantitative analysis of sedimentation profiles we calculated the $S_{1/2}$ values described by Veatch and Okada.

The $S_{1/2}$ values of unirradiated cells were 21.10, and after 200 Gray irradiation 11.35, due to the original incidence of single-strand breaks. The presence of pKM101 did not influence these values in either cases. This means that pKM101 had no effect on the rise of single-strand breaks in DNA.

During a post-irradiation incubation period at 37°C for 60 min the $S_{1/2}$ value of the wild type strain increased from 11.35 to 19.22, that of the recB$^-$ from 11.50 to 15.23, while the $S_{1/2}$ value of the recA$^-$ mutant did not change owing to the lack of repair of single-strand breaks.

pKM101 plasmid markedly increased the $S_{1/2}$ value in wild type strain and in recB$^-$ mutant, while it had no effect on $S_{1/2}$ in recA$^-$ cells, during this post-irradiation incubation period. Thus the effect of pKM101 on the repair of single-strand breaks in DNA proved to be dependent on recA$^+$ genotype.

Nalidixic acid at 100 µg/ml concentration inhibited the repair of single-strand breaks in both wild type and recB$^-$ mutant cells harbouring pKM101 plasmid.

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INTRODUCTION

Certain R plasmids, including R46 and its derivative pKM101 have been shown to increase the survival and mutagenesis of bacteria that were exposed to ultraviolet light or methyl-methan sulphonate (Walker, 1977). R46 also protected its host against DNA damaging agents such as nitrofurantoin and bleomycin. In our former studies in E. coli K12, both plasmids enhanced bacterial survival after ^{60}Co-gamma irradiation (Francia et al., 1985). This effect proved to be dependent on recA$^+$ genotype but not on recB$^+$, recB$^+$ recC$^+$ and recF$^+$ genotypes.

Ionizing irradiation normally causes single and double strand breaks in the desoxyribosephosphate diester backbone of DNA, with 15 single-strand breaks for each double-strand. Nitrofurantoin and bleomycin also cause single-strand breaks which are produced either directly as a consequence of their primary action on DNA, or indirectly by host repair activity.

The R46 and pKM101 plasmid mediated protection against ^{60}Co-gamma irradiation could therefore involve plasmid-gene products in their repair of single-strand gaps or breaks.

The similar protective effect of R46 and pKM101 against UV and ^{60}Co-gamma irradiation supports the possibility that the enhanced survival, conferred by these plasmids, is due to altered processing of gamma ray-induced damages, other than DNA strand breaks. It is well-known that besides the strand breaks, ionizing irradiation also causes damages of heterocyclic bases.

It was in this frame of reference that we decided to examine the effect of pKM101 plasmid on the repair of single-strand breaks in DNA of E. coli K12 cells induced by ionizing irradiation. The increase of single-strand breaks and the rejoining was studied by alkaline sucrose gradient method. At the same time we wanted to compare the effect of nalidixic acid on the repair of single-strand breaks induced by ionizing irradiation in wild type and rec$^-$ mutants E. coli K12 cells, harbouring pKM101 plasmid. These experiments are described in the present paper.

MATERIALS AND METHODS

Nalidixic acid was supplied by MERCK.

Bacteria and bacterial media

E. coli K12 AB 1157 and its recA$^-$ and recB$^-$ mutants harbouring either R-factor pKM101 or not (with or without) were used as tested strains. The properties, sources of these strains were described in our previous paper (Francia et al., 1985).

Determination of single-strand breaks

Alkaline sucrose gradient studies were based on the method described by McGrath and Williams (1966). For the quantitative analysis of the curves we have calculated the $S_{1/2}$ values described by Veatch and Okada in 1964 (1969). Escherichia coli cells were labelled in their DNA by inoculating stationary-phase bacteria into prewarmed "GS" medium (Ganesan, Smith, 1968), containing ^{3}H-methyl-thymine (7.4 kBq/ml) medium and the required amino acids (0.15 g/l). The size of the inoculum was adjusted so as to allow 3 to 4 generations of growth in the label-containing media before harvesting the cells in exponential growth at $2x10^8$ cells/ml. The cells were filtered on Sartorius, 0.45 um pore size, washed and resuspended in "GS" medium, without supplements required and then they were irradiated. Samples with supplements required, were incubated at 37^{o}C for 60 min (in certain cases in the presence of nalidix acid (NA) at 100 ug/ml concentration); and then were transferred to a chilled centrifuge tube. All samples were pelleted, washed twice in 0.05 mol/l Tris-HCl (pH 8.1) and transformed into spheroplasts 0.3 ml samples by the method of Rupp and Howard-Flanders (1968). Approximately $5x10^6$ spheroplasts were given into tubes containing 36 ml of linear gradient of 5-20 per cent alkaline sucrose (w/v; pH 12.0). The gradient tubes were centrifuged in SW rotor for 240 min at 25000 rev/min at 18 ^{o}C (Beckman-Spinco, Model L3-50). After the run, about 36 fractions of 1.04 ml were collected. 0.2 ml of each fraction was pipetted into filter discs (Whatman 3MM paper). Then the discs were washed twice in 5 per cent trichloroacetic acid and each was washed once in ethanol and acetone. After drying, the filters were given into 10 ml of the

liquid scintillant (4 g PPO and 0.1 g POPOP per 1 lit. toluene), and radioactivity was measured in Nuclear Chicago Isocap Scintillation Spectrometer (Model 300).

Each sample was centrifuged in triplicate, and the density gradient profile was fitted by eye to the three sets of points which generally coincided with the same continuous curve.

Irradiation

Irradiation was carried out by using ^{60}Co source (200 Gray) dose rate: 15.42 Gray/min at 4°C.

RESULTS

Fig. 1 reveals the sedimentation patterns of DNA of E. coli K12 AB 1157 cells, without, and after, irradiation with 200 Gray which are dependent on the incubation period after irradiation. The shift in pattern can be associated to DNA single-strand breaks induced by irradiation.

The curves were quantitatively analyzed by calculating the $S_{1/2}$ values (Ganesan, Smith, 1968). The $S_{1/2}$ is the distance in fractions, beyond which half of the mass of DNA sediments. It is expressed in fraction number from the top, and indicated on the figures by vertical bars.

It can be seen than the $S_{1/2}$ value of the unirradiated control was 21.10 and, after 200 Gray dose of irradiation, it was found to be 11.35. A post-irradiation incubation at 37°C for 60 min increased the $S_{1/2}$ value from 11.35 to 19.22. Fig. 2 shows that the presence of R-factor pKM101 hardly influenced the sedimentation profiles of DNA of irradiated and unirradiated E. coli K12 AB 1157 cells, the $S_{1/2}$ values were 13.18 and 22.35. The repair of the single-strand breaks in cells harbouring pKM101, however, seemed to be more complete, with a $S_{1/2}$ value of 22.0.

The curves of Fig. 3 and 4 present the sedimentation profiles of DNA of E. coli K12 JC2926 (recA13) and E. coli K12 JC5519 (recB21) cells, in the absence of pKM101, without, and after irradiation with the dose of 200 Gray.

The sedimentation patterns and the $S_{1/2}$ values of unirradiated control and the irradiated cells in both strains were similar to the curves shown in former figures. The $recA^-$ mutant, however, has no capacity for single-

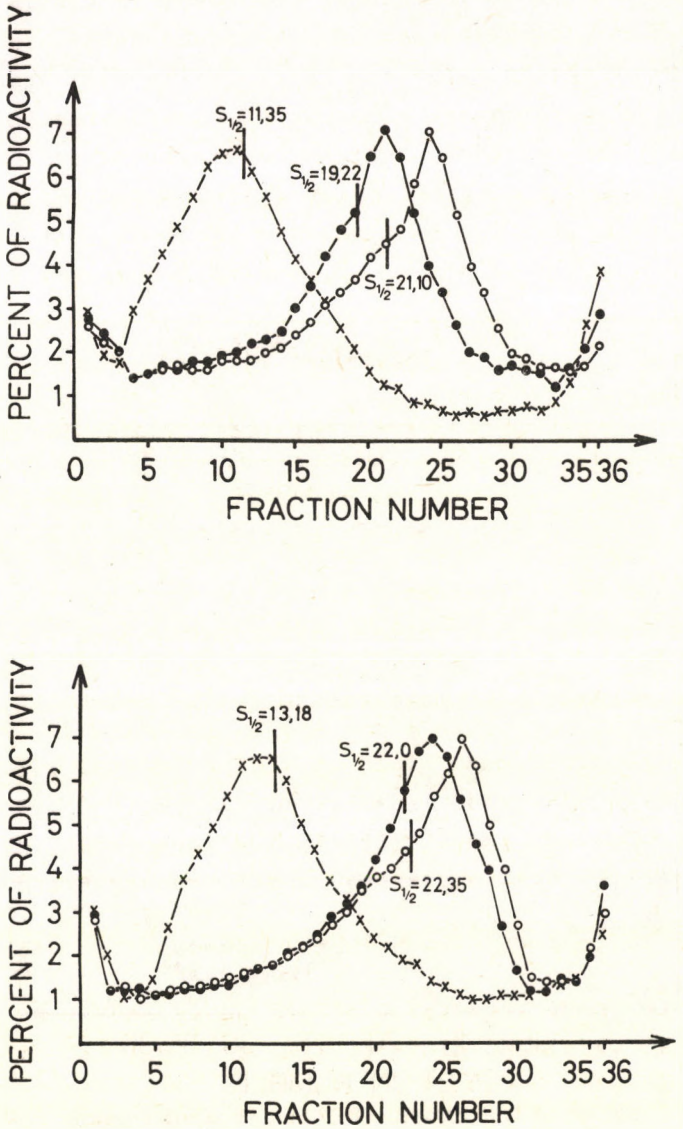
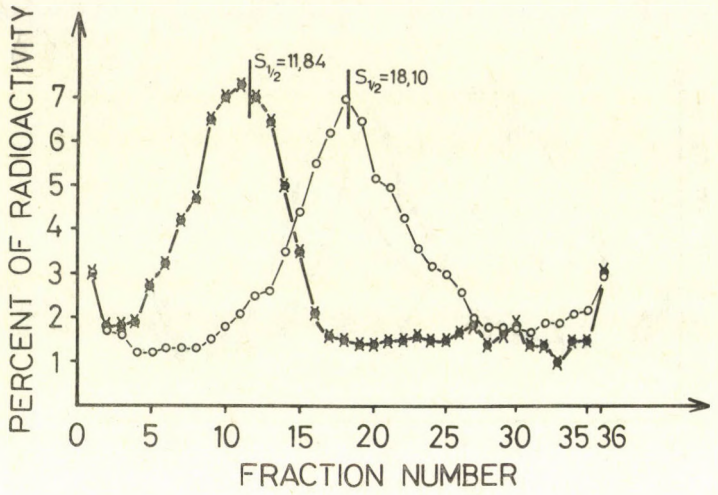


Fig. 1 and 2 Sedimentation profiles of DNA of E. coli K12 AB1157 cells without (Fig. 1) and with (Fig. 2) pKM101 in alkaline sucrose.
Unirradiated control: o——o; irradiated with 200 Gray without post-irradiation incubation: x——x; irradiated with 200 Gray and incubated for 60 min at 37°C after irradiation: ●——●.



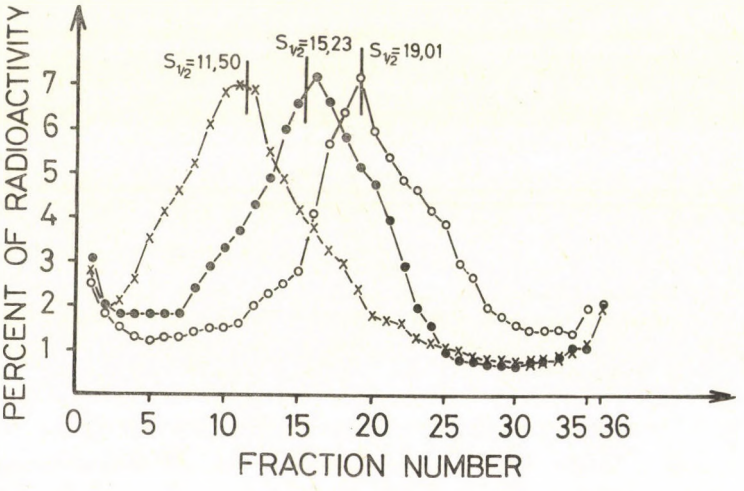


Fig. 3 and 4 Sedimentation profils of DNA of E. coli K12 recA13 (Fig. 3) and E. coli K12 recB21 (Fig. 4) cells in alkaline sucrose. Unirradiated control: o—o; irradiated with 200 Gray without post-irradiation incubation: x—x; irradiated with 200 Gray and incubated for 60 min at 37°C after irradiation: ●—●.

strand repair, therefore the $S_{1/2}$ value in this strain remained around 12 (Fig. 3) while the recB$^-$ mutant showed a slight repair, with a $S_{1/2}$ value of 15.23 (Fig. 4), after a 60 min post-irradiation incubation period at 37^{o}C.

Fig. 5 and 6 show the sedimentation profiles of the above two rec$^-$ mutants in the presence of pKM101, without and after irradiation with the same dose of 200 Gray.

pKM101 R-factor had no influence on the sedimentation pattern of recA$^-$, as the $S_{1/2}$ value remained 11.96 (Fig. 5), however, in recB$^-$ mutant (Fig. 6) the above R-factor increased the $S_{1/2}$ value from 11.18 to 14.43.

Fig. 7 and 8 indicate the effect of nalidixic acid at 100 µg/ml concentration on the repair of single-strand breaks in DNA of E.coli K12 AB1157 and E.coli JC5519 (recB21) harbouring pKM101. Nalidixic acid in both cases inhibited the DNA repair, the $S_{1/2}$ values were found to be: for K12: AB1157 cells without irradiation (Fig. 7): 22.35; after 60 min post-irradiation incubation at 37^{o}C in the absence of nalidixic acid: 13.18; and in its presence: 13.84; and for recB21 cells (Fig. 8) without irradiation: 19.01; after 60 min post-irradiation incubation at 37^{o}C in the absence of nalidixic acid: 11.50; and in its presence: 12.09.

DISCUSSION

In our earlier experiment, R46 and pKM101 plasmids enhanced bacterial survival after ^{60}Co-gamma irradiation in E.coli K12 cells. We supposed that R46 and pKM101 plasmid mediated protection against ^{60}Co-gamma irradiation involved plasmid gene products in their repair of single-strand breaks. There was also the possibility that the enhanced survival attributed to these plasmids was due to the repair of gamma ray induced damages other than DNA strand breaks.

It is a well-known fact that ionizing radiation induces different lesions in the genetic material of cells, including single- and double strand breaks as well as damages to sugar and base structures. Among the damages, the single-strand breaks are most easily repairable. The reconstruction of the radiation induced single-strand breaks has been shown to occur in many organisms including E. coli K12 cell.

In our experiments presented here we wanted to clarify the above

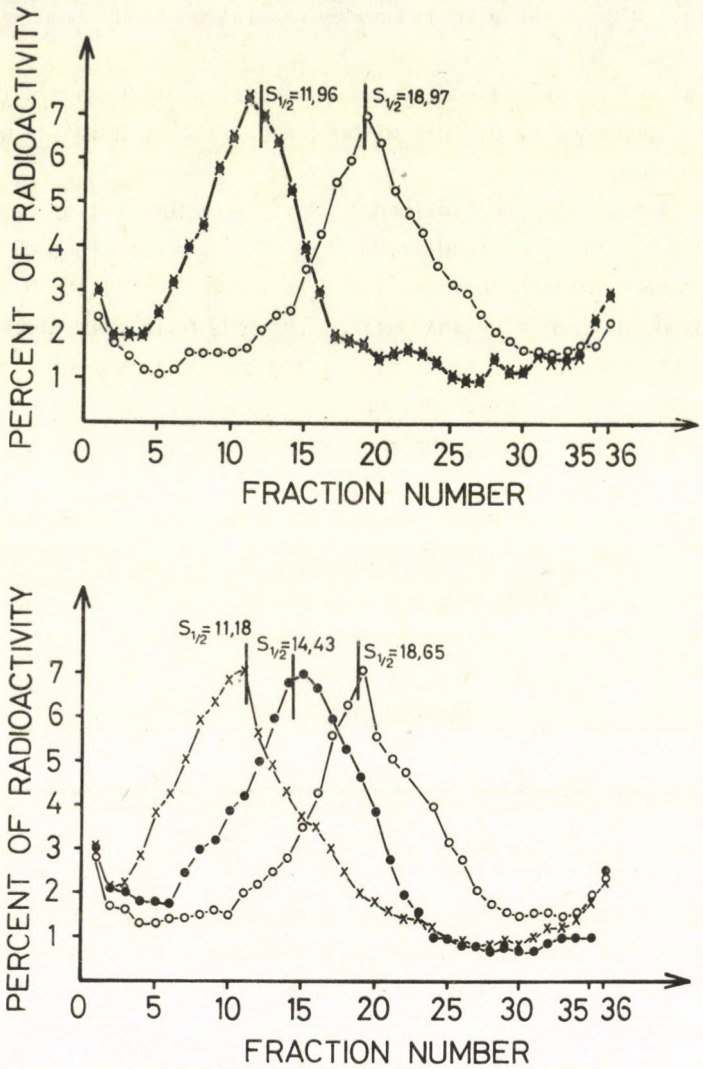


Fig. 5 and 6 Sedimentation profiles of DNA of E. coli K12 recA13 (Fig. 5) and E. coli K12 recB21 (Fig. 4) cells with pKM101 in alkaline sucrose

Unirradiated control: o—o; irradiated with 200 Gray without post-irradiation incubation: x—x; irradiated with 200 Gray and incubated for 60 min at 37°C after irradiation: ●—●.

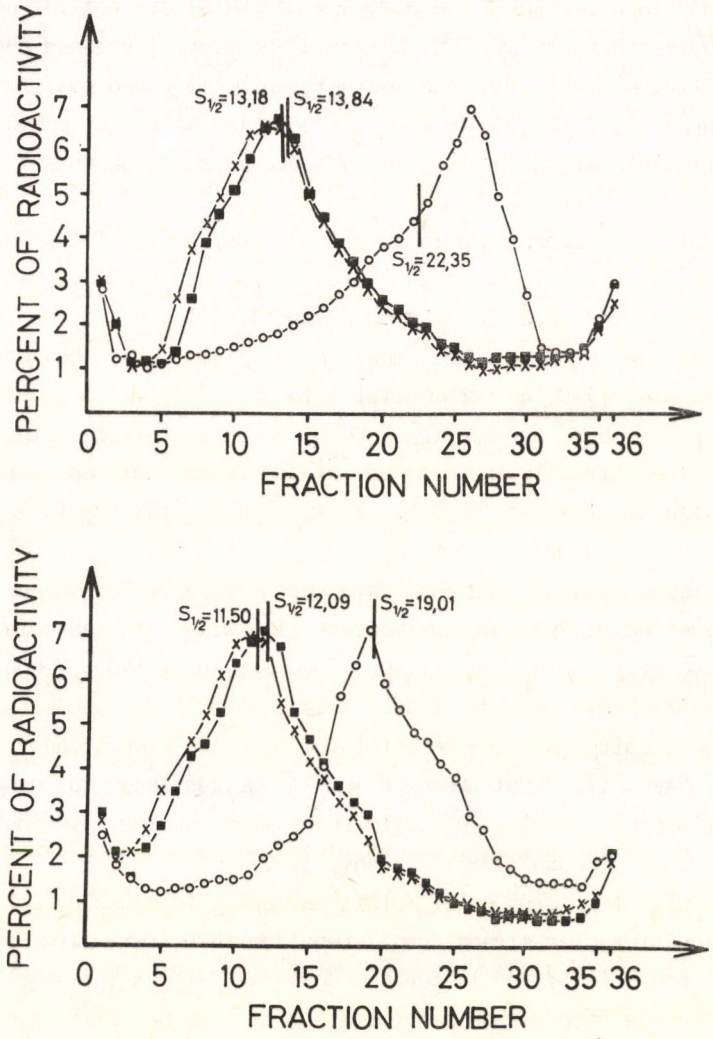


Fig. 7 and 8 The effect of nalidixic acid at 100 µg/ml concentration on the repair of radiation induced single-strand breaks in DNA of E. coli K12 AB1157 cells (Fig. 7) and E. coli K12 recB21 cells, harbouring pKM101 (Fig. 8).
Unirradiated control: o—o; irradiated with 200 Gray without post-irradiation incubation: x—x; irradiated with 200 Gray, treated with 100 µg/ml nalidixic acid and incubated for 60 min at 37°C after irradiation: ●—●.

question by examining the effect of pKM101 plasmid directly on the repair of single-strand breaks in DNA induced by ^{61}Co-gamma irradiation.

According to our results the presence of pKM101 did not influence the amount of single-strand breaks. This plasmid, however, increased the repair in wild type strain and in its recB$^-$ mutant, while it could not promote the reparation processes in recA$^-$ cells. Thus the effect of pKM101 on the repair of single strand breaks in DNA proved to be dependent on recA$^-$ genotype.

The alkaline sucrose gradient method was chosen for studying DNA single-strand rejoining. The reason for this was that generally the sedimentation technique proves to be more sensitive to follow the rejoining processes than the other methods (McGrath, Williams, 1966; Körner et al., 1977; Koval et al., 1979; Ahnström et al., 1978).

According to our presumption pKM101 plasmid affects the repair mechanism, either directly inducing one of the repair enzymes, taking part in different repair types, or indirectly, influencing the regulation of DNA repair.

The excision repair sequence involves a few enzymes: the uvrA, uvrB and uvrC endonucleases, DNA polymerase I. with its associated 5'-exonuclease activity and DNA ligase (Sancar, Rupp, 1983; Yeung et al., 1983; Tait et al., 1974; Youngs et al., 1974).

Excision repair can also be initiated by a two-step process in which the damages base is first removed by a glycosylase to generate an apurinic/apyrimidinic (AP) site, followed by an exonucleolitic incision by an AP endonuclease (Duncan, 1981; Lindahl, 1982).

In E. coli four classes of mutations appear to cause deficiences in postreplication (daghter-strand gap) repair, recA, ruv, lexA, and recF. However, the products of the latter two genes may affect the regulation of the repair process rather than participating at a mechanistic level (Rupp, Howard-Flanders, 1968; Rupp et al., 1971; Walker, 1985). At the same time, the recombinational repair of double strand breaks, introduced by ionizing radiation or mitomycin C, requires the products of the recN and recA genes, and this seems to be a recB-dependent process that reapirs double strand breaks in UV-irradiated DNA (Krasin, Hutchinson, 1981; Picksley et al., 1984).

Finally the error-prone repair or "SOS processing" (Walker, 1984,

1985; Witkin, 1976) requires the products of the umuD and umuC genes, since inactivation of either of these renders cells nonmutable by UV and many chemicals (Elledge, Walker, 1983; Kato, Shinura, 1977). Furthermore, the recA gene product plays some additional role in SOS processing besides being involved in the transcriptional regulation of the umuDC operon (Walker, 1984; Witkin, Kogoma, 1984).

The expression of many E. coli DNA repair genes is induced by DNA damage. Exposure of E. coli to agents that damage DNA can induce the expression of at least three independent regulatory networks, among which the SOS (Walker, 1984, 1985; Witkin, 1976; Little, Mount, 1982) is the most important.

The SOS responses are induced by various treatments or conditiones that damage DNA or block DNA replication. DNA repair genes, regulated by the SOS circuitry, include uvrA, uvrB, uvrC, uvrD, recN, ruv, umuD, umuC an recA (Hori et al., 1981; Little, 1984; Little et al., 1980). LexA serves as a repressor of every chromosomal din (damage-inducible) gene, identified to date, and is cleaved as the result of the SOS inducing treatment.

The gene map of pKM101 has been made, the loci of genes (e.g. mucA, mucB and others) are determined. We think that mucA and mucB gene products having similar functions as umuB and umuC gene products which enhance the abiliy of cells to induce SOS processing thus can indirectly promote DNA repair process as well. It is also possible that among the till not determined gene products of pKM101 there is one (or more) which is homologous with one of the DNA repair enzymes and thus directly can take part in DNA repair.

Because of the $recA^+$ dependence of the effect of pKM101 on single-strand repair it seems more likely that the plasmid specially influences the regulation of DNA repair, namely enhances the ability of cells to carry out SOS processing.

According to Eberle's and Masker's results nalidixic acid, at 20 µg/ml concentration, inhibited both semi-conservative DNA replication and repair DNA synthesis after UV irradiation.

In our experiments nalidixic acid blocked DNA repair and the effect of pKM101 plasmid on the repair of single-strand breaks but only in 100 µg/ml concentration. Thus, the activity of nalidixic acid cannot be considered as a specific inhibitory effect on DNA polymerase I or on other repair enzymes.

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PHOTOELECTRIC RESPONSE SIGNALS OF BACTERIORHODOPSIN CONTAINING 13-CIS RETINAL

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SUMMARY

The electric response signal of dark adapted bacteriorhodopsin excited by a laser flash was studied. By a subtraction method the electric signal corresponding to the bacteriorhodopsin containing 13-cis retinal could be separated. This electric signal contains three components, corresponding to three steps in the charge movement induced by the laser flash. The lifetimes of these steps are correlated with the photocycle of the bacteriorhodopsin containing 13-cis retinal.

INTRODUCTION

It is well known that the light- and dark- adapted forms of bacteriorhodopsin behave differently (Stoeckenius et al., 1979). While bR^{LA} contains only all-trans retinal, the composition of bR^{DA} is about 50 per cent all-trans and 50 per cent 13-cis retinal in thermal equilibrium. Both forms have different photocycles. Only the all-trans retinal-containing bR takes part in the proton pumping and has in the photocycle the M form. The process of light adaptation can be followed by monitoring the concentration of the M form. It was shown that the ratio of its concentrations in bR^{LA}

ABBREVIATIONS: bR^{LA}, light-adapted bacteriorhodopsin; bR^{DA} dark-adapted bacteriorhodopsin; $bR^{13\text{-}cis}$, bacteriorhodopsin containing 13-cis retinal.

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and bR^{DA} is two (Ohno et al., 1977). The same ratio was observed in the amplitude of the photoelectric signals corresponding to the M form during dark adaptation of the sample (Fahr, Bamberg, 1982). The process of light and dark adaptation is influenced by the water content of the dried purple membrane samples (Korenstein, Hess, 1977; Váró, 1981).

While the photocycle of the bR containing 13-cis retinal is known (Stoeckenius et al., 1979), few works have referred to the existence of an electric signal associated with it (Drachev et al., 1978). It has been shown that the first negative electric signal is larger in bR^{DA} than in bR^{LA} after flash excitation. The sign of the electric signals is related to the known signals of bR^{LA} (Keszthelyi, Ormos, 1980). This result indicated that intramolecular charge motions could be associated with the photocycle of $bR^{13\text{-cis}}$.

Here a detailed study of the electric signals produced by laser flash excitation in $bR^{13\text{-cis}}$ is presented. In addition to the previously found negative signal (Drachev et al., 1978) two more signals were registered which may be associated with known transitions in the $bR^{13\text{-cis}}$ photocycle.

MATERIALS AND METHODS

The purple membrane fragments used in the preparation of the dried oriented samples were obtained by the standard procedure from Halobacterium halobium strain ET 1001 (Oesterhelt, Stoeckenius, 1974). The preparation of the dried samples has been described elsewhere (Váró, 1981; Váró, Keszthelyi, 1983). The measuring system of the photoelectric signals, reported by Váró and Keszthelyi (1983), was only slightly modified: the temperature of the sample was controlled in the range of $-25^{o}C$ to $+30^{o}C$ with Peltier cells and measured by a thermocouple. The sample was incubated in the sample holder in a closed atmosphere, the relative humidity of which was set to 0.85 with a saturated solution of KCl. This humidity assured the possibility of the light-dark adaptation (Korenstein, Hess, 1977; Váró, Keszthelyi, 1983).

The flash intensity of the exciting Opton dye laser (pulse length 1μs, λ = 590 nm) was reduced by neutral density filters to 10-20 $\mu J/cm^2$ to avoid light adaptation during excitation. All the data reported originate from single recordings, i.e. the signals were not averaged.

Dark adaptation of the sample was assured by keeping it overnight in the dark. Light adaptation was made by a mercury lamp (HBO 200, Carl Zeiss) with heat and green light glass filters (λ_{max} = 550 nm). The signals were computer analyzed.

RESULTS AND DISCUSSION

The photoelectric signals were measured first with the time constant of the electric circuit RC = 2 ms (shunting resistance R = 10 MΩ , capacitance of the sample C = 200 pF). This circuit measured the photovoltage of the electric signals which were faster than RC (Váró, 1981; Ormos et al, 1983). The positive maximum of the photoelectric signal in this case was proportional to the concentration of the M form. The maximum was twice as large in the light adapted form as in the dark adapted one. The two signals were normalized by multiplication of the dark adapted signal by two (Fig. 1).

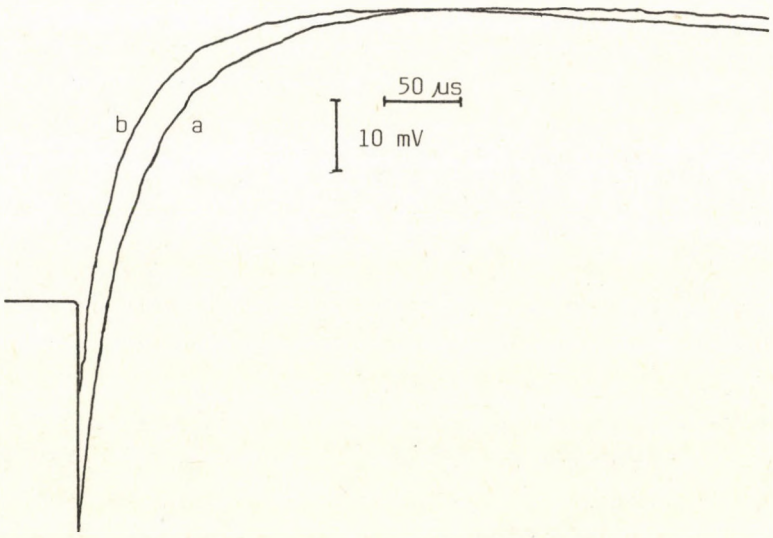

Fig. 1 The photoelectric signal of the bR^{DA} (a) and bR^{LA} (b) normalised to the positive maximum. The measuring conditions are: circuit resistance 10^7 Ω sample capacitance 200 pF, temperature 22°C, relative humidity 0.85.

The calculated difference signal:

$$bR^{13\text{-cis}} = 2 \times bR^{DA} - bR^{LA}$$

shown in Fig. 2 appears quickly and decays slowly. By separate measurements (data not shown) it was determined that the appearance of the signal is faster than 20 ns. The decay, which goes nearly to zero voltage, was decomposed into two exponentials with lifetimes 32 μs and 146 μs. A charging and discharging phase in a photovoltage means at least two components of charge displacement.

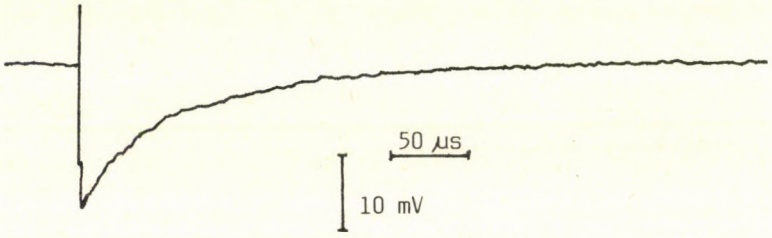

Fig. 2 The photoelectric signal of the $bR^{13\text{-cis}}$ obtained by subtraction of curve **b** from **a** of Fig. 1.

The electric signals characterizing the $bR^{13\text{-cis}}$ photocycle may be further studied by observing the photocurrent (Váró 1981; Ormos et al., 1983). By changing the shunting resistor to R = 1 kΩ , the time constant of the measuring circuit was altered to (RC)' = 200 ns, enabling photocurrent signals over the microsecond time scale to be measured. At low temperatures a new electric signal appeared in the dark adapted sample, which did not exist in the light adapted one. In Fig. 3 signal **a** is twice the bR^{DA} signal while **b** is the recorded bR^{LA} signal. The difference between 2 x bR^{DA} and bR^{LA} is the $bR^{13\text{-cis}}$ photocurrent signal (Fig. 4).

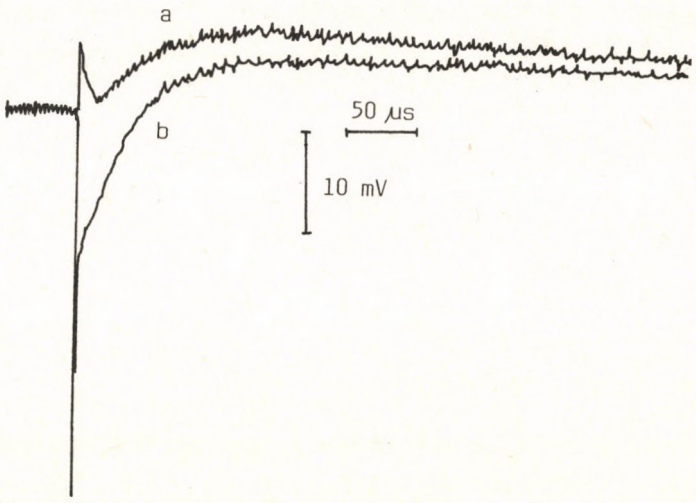

Fig. 3 The photoelectric signal of the bR^{DA} (a) and bR^{LA} (b) normalized. The measuring conditions are: circuit resistance 1 kΩ, sample capacitance 200 pF, temperature -13°C, relative humidity 0.85.

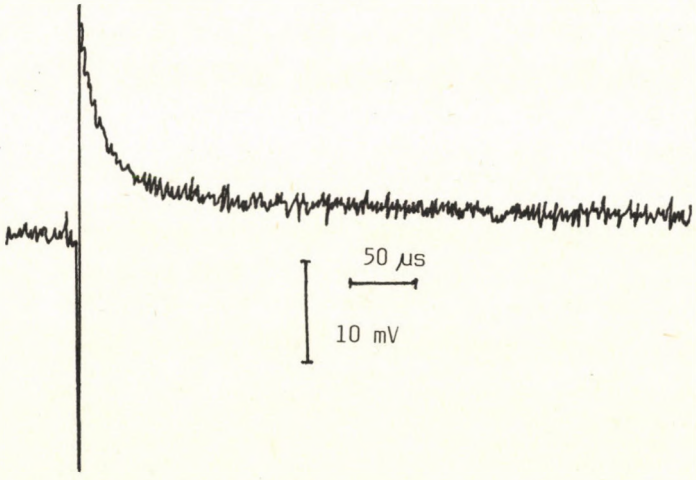

Fig. 4 The photoelectric signal of the $bR^{13\text{-cis}}$ obtained by subtraction of curve **b** from curve **a** of Fig. 3.

Three distinctly different electric signals appeared. There is a large rapidly rising negative signal which decays with the time constant of the electronics ((RC)' = 200 ns). The second is a positive signal with a short lifetime going over to a long living positive signal of small amplitude. The lifetime of the fast positive signal was measured at different temperatures and the Arrhenius parameters were determined: the preexponential factor A = 3×10 s^{-1}, and the activation enthalpy H = 56 kJ/mole. Using these parameters the lifetime of this electric signal is $\tau_2 \simeq 350$ ns at room temperaature (T=20°C).

The photocurrent measurement confirmed and extended the results of the photovoltage measurement. The large negative signal is the charging phase of the photovoltage. It corresponds with great certainty to the $bR^{13\text{-cis}}$-C^{x} transition of the photocycle (Stoeckenius et al., 1979). The two positive signals discharge the negative photovoltage. Only the slow component appears in Fig. 2. The rapidly discharging signal has a small charge movement and is very fast at room temperature, and hence can not be seen in Fig. 2. The fast discharging signal is probably associated with the C^{x} - C^{610} transition. The slow discharging signal in Fig. 2 corresponds to the slow positive current signal in Fig. 4. This can be an event during the long-lasting C^{610} - $bR^{13\text{-cis}}$ transition.

It may be seen in Fig. 2 that the signal almost decays to zero in $bR^{13\text{-cis}}$ molecules while the photovoltage rises to positive values in bR^{LA} molecules, i.e. in all-trans bacteriorhodopsin (Keszthelyi, Ormos, 1980). This observation is in agreement with the known fact that bR^{13cis} molecules do not pump protons under illumination.

Electric signals were recorded in flash-excited 13-cis bacteriorhodopsin molecules. Because the signals correspond to internal charge motions we may state that a very fast ($\tau_1 < 20$ ns) backward charge motion is followed by a fast ($\tau_2 \simeq 350$ ns) and a composite slow ($\tau_{3'} = 32$ µs, $\tau_{3''} = 146$ µs) component. The algebraic sum of the charge motions is apparently zero (this needs, however, further confirmation by following the signals in the ms time range). These time constants of the electric signals may be correlated with the known transitions in the photocycle of bR.

ACKNOWLEDGEMENTS

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DISTRIBUTION OF Ca IN DIFFERENT REGIONS OF HEART MUSCLE CELL

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SUMMARY

Electron microscopic autoradiographic investigations to study the localization of Ca in muscle were performed using heart muscle. The distribution of autoradiographic grains, originating from Ca-45 were compared above myofibrils and interfibrillar spaces of the ventricle, auricle and sinus node of the heart. Our aim was to get new data on the role of Ca in the mechanical activity of muscle with special consideration of the Ca content of interfibrillar spaces. The different distribution of grains in the muscle fibrils and interfibrillar spaces of various parts of the heart can be in connection with the different activities of these parts. The more Ca content in myofibrils of ventricle cells may be in connection with its greater mechanical activity and its work.

INTRODUCTION

One of the aims of the research work going on in this Institute is to clarify the localization of inorganic ions, and the changes of these substances during muscular activity. (Ernst, 1963; Tigyi, 1968; Tigyi-Sebes, 1962; Nesterov, Tigyi-Sebes, 1965; Kállay, Tigyi-Sebes, 1968). One of the most important material of these inorganic elements is the Ca, which serves as a coupling between the excitation and contraction.

Many researchers investigated the role of Ca in muscular activity (Lánczos, 1935; 1936; Heilbrunn and Wiercinsky, 1947; Bozler, 1952; A. Weber, 1959, 1961; Ebashi, 1961, 1962). Some of them tried to approach the question from the field of Ca localization (Onishi and Ebashi, 1963; Ebashi, 1964; Winegrad, 1965, 1965a).

We have also investigated the distribution of Ca in skeletal muscle

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(Kállay, Tigyi-Sebes, 1973), and the change of its distribution in different circumsatances (Tigyi, Kállay, 1978; Kállay et al., 1978; Tigyi et al., 1980-81).

It is better to investigate the localization of Ca in heart muscle than in skeletal muscle because it has a wider sarcoplasmic reticulum and it contains cell parts other than myofibrils, as well. So, we performed our investigations with this specimen.

METHODS

The heart muscles of frogs (Rana esculenta) were used. The excised hearts were prepared by Straub's cannula with inactive Ringer solution. After 5 to 10 minutes of preparation the inactive Ringer solution was changed to a Ringer solution containing 1 to 2 MBq/ml of Ca-45 in the form of $CaCl_2$. This active solution was changed in every 5 minutes through 2 hours. During this process the heart kept beating normally at room temperature.

At the end of the 2nd hour the cannula was taken off the heart which was cut into 3 pieces, the ventricle, the auricle and the sinus node. These pieces were fixed in vapour of 5 per cent of OsO_4 solution for 1 hour. The fixed pieces of heart were dehydrated in an alcohol series, embedded and cut for electron microscopic investigation.

The specimen on grids were fixed to a slide with a thin film and they were covered with a twice diluted solution of Ilford L-4 emulsion. For preparing the emulsion film the loop method of Caro and Tubergen (1962) was used. After 40 to 50 days exposure time at 2^{o}C, the preparations were developed in Ansco 47/a developer (at 20^{o}C for 7 minutes), fixed (for 7 minutes) and rinsed in twice-distilled water (for 15 minutes). The whole process was performed in a dark room using Ilford Darkroom Safelight S 902.

After drying in air the grids were carefully removed from the slides. The cuts were painted with phosphorous-wolframic acid and examined in electron microscope. The autoradiographs made from each part of the heart were evaluated according to grain count. The resolving power of the method is 0,1 μm like the diameter of the grains of Ilford L-4 emulsion.

RESULT

60 autoradiographs were evaluated from each part of heart muscle, the ventricle, sinus node, and auricle (Figs. 1, 2 and 3).

Table 1 shows the grain densities in 100 μm^2 areas of the myofibrils and the sarcoplasmic reticulum of the different parts of heart. The grain density is a little higher above the myofibrils than above the sarcoplasmic reticulum in the case of ventricular muscle. The densities are totally equal in the case of muscle of sinus node, and essentially lower values were found above the myofibrils of auricular muscle than above its sarcoplasmic reticulum. The grain density above the myofibrils is the highest in the ventricular muscle and the lowest in the auricular muscle.

Table 1 Grain density above the myofibril and sarcoplasmic reticulum of different parts of heart muscle

	Grain density $\frac{N}{100\ \mu m^2}$	
	above myofibril	above sarcoplasmic reticulum
Ventricle	14.68 ± 3.83	13.22 ± 3.63
Sinus node	13.10 ± 3.61	13.10 ± 3.61
Auricle	10.04 ± 3.14	18.76 ± 4.33

At the interfibrillar spaces grains could be found over all of the cell components. (Fig. 4, 5, 6).

The distribution of these grains among some cell components is shown in Table 2. As it can be seen, more than the half of grains is localized

above the tubular system of sarcoplasmic reticulum. Only 1.5 to 2 per cent of grains was not in contact with these cell components. This fraction was above the cell membrane or collagen fibres or other components.

Table 2 Percentile distribution of grains above the different cell components

	Percentile distribution of grains		
	above tubular system	above mitochondria	above nucleus
Ventricle	60	30	8
Sinus node	58	30	10
Auricle	62	26	10

DISCUSSION

In our previous works we described the localization of Ca in skeletal muscle (Kállay, Tigyi-Sebes, 1975; Kállay et al., 1978). Then we published that there may be a little uncertainty about the origin of beta particles exposing the grains above the sarcoplasmic reticulum because the width of it was too small.

The heart muscles having more wide sarcoplasmatic reticulum and other components of muscle cells, are better to investigate the distribution of Ca not only in the myofibrils of the muscle but in the sarcoplasmic reticulum, too.

From these experiments some conclusion can be drawn concerning the localization of Ca in heart muscle and the dynamics of its movement in heart muscle cell. The fact that we found radioactive Ca (Ca-45) in every

examined region of heart muscle cell after two hours working refers to a rather intensive Ca mobility in the cell components. The Ca-40 exchange for Ca-45 may happen across the tubular system. Considering, however, that the frog heart contains very few sarcoplasmic reticuli this exchange may happen through the cell membrane, too. The Ca found in the myofibrils refers to its role in muscular contraction. That Ca was slightly higher in the ventricular muscle (which does the most of the work of the heart) than in the auricular muscle.

According to the literature the Ca is stored in the tubular system of sarcoplasmic reticulum of muscle. (Winegrads, 1965; Jöbsis, 1967). The 60 per cent of grains found in our experiments above the tubular system of sarcoplasmic reticulum seems to confirm this view.

The authors thank professor J. Tigyi for his help in connection with this work and the co-workers of the Central Laboratory for the technical helps.

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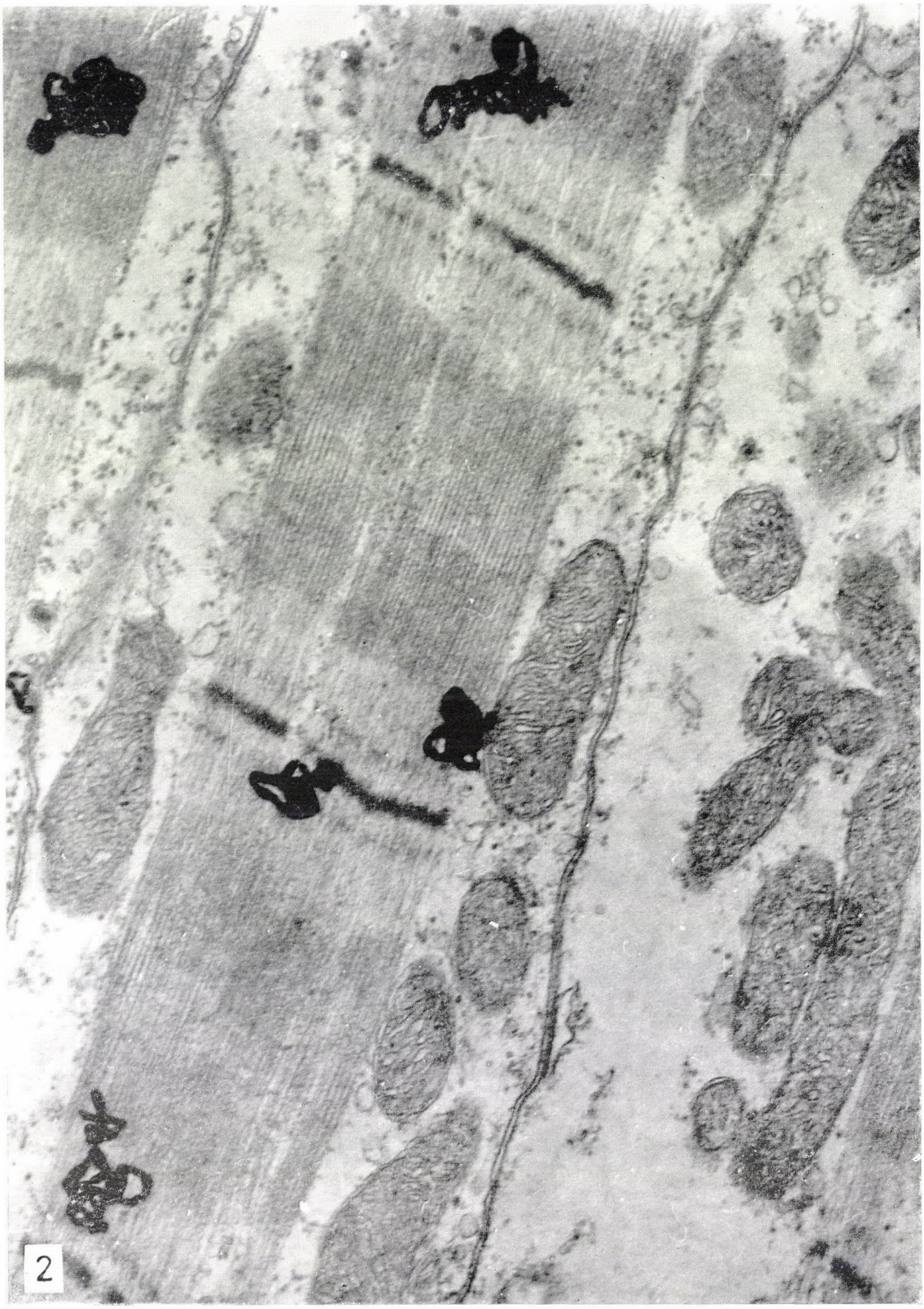
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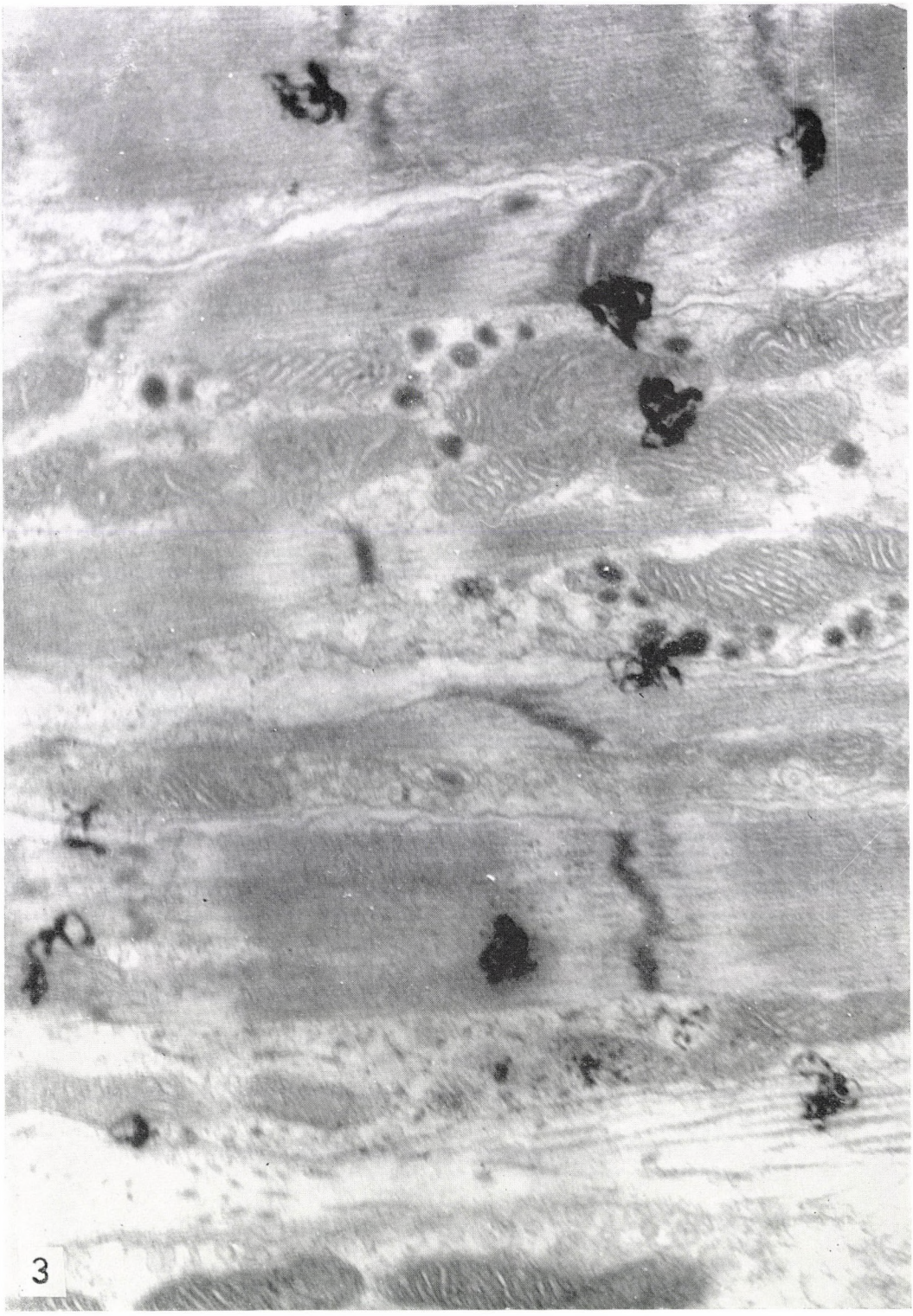

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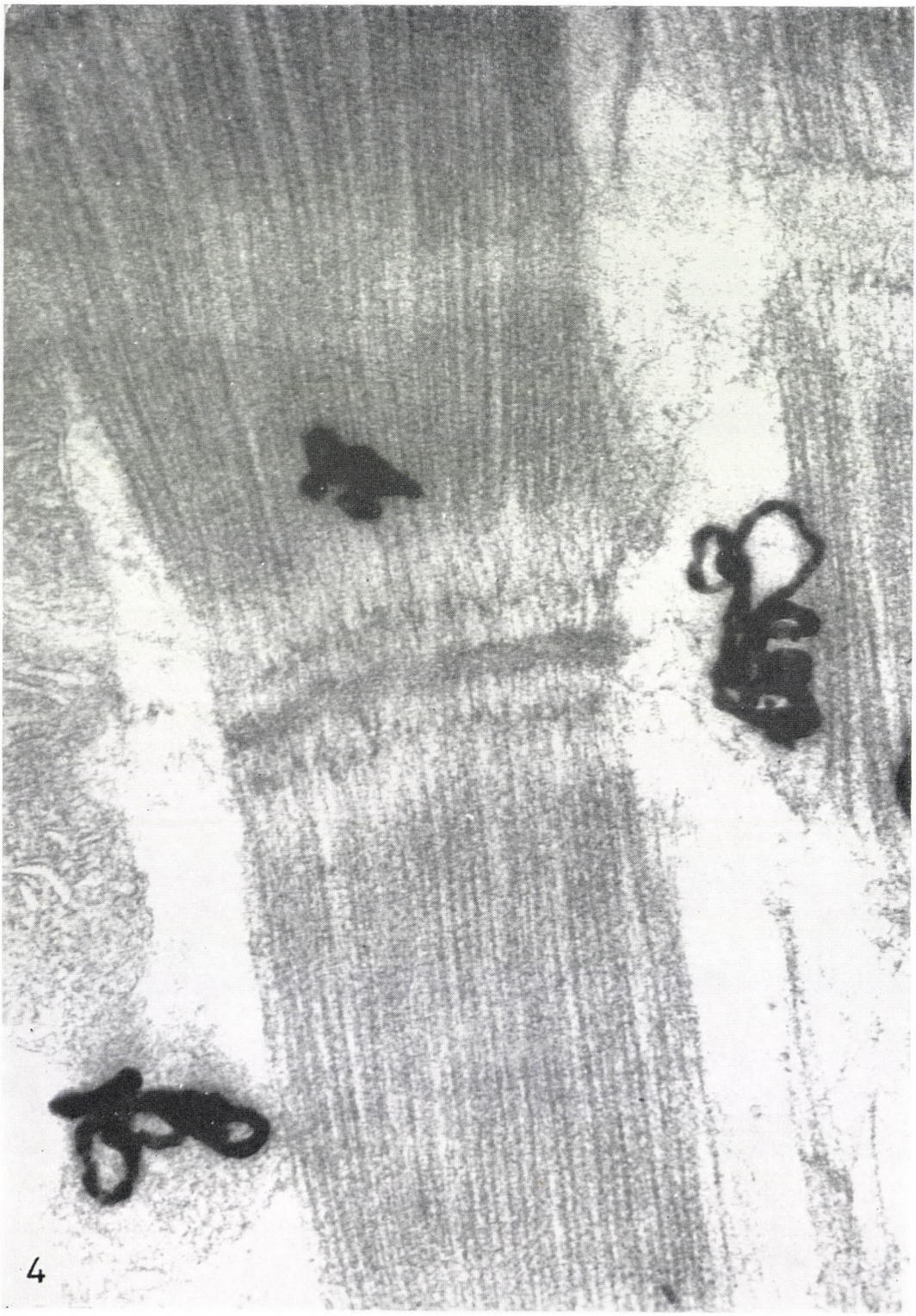

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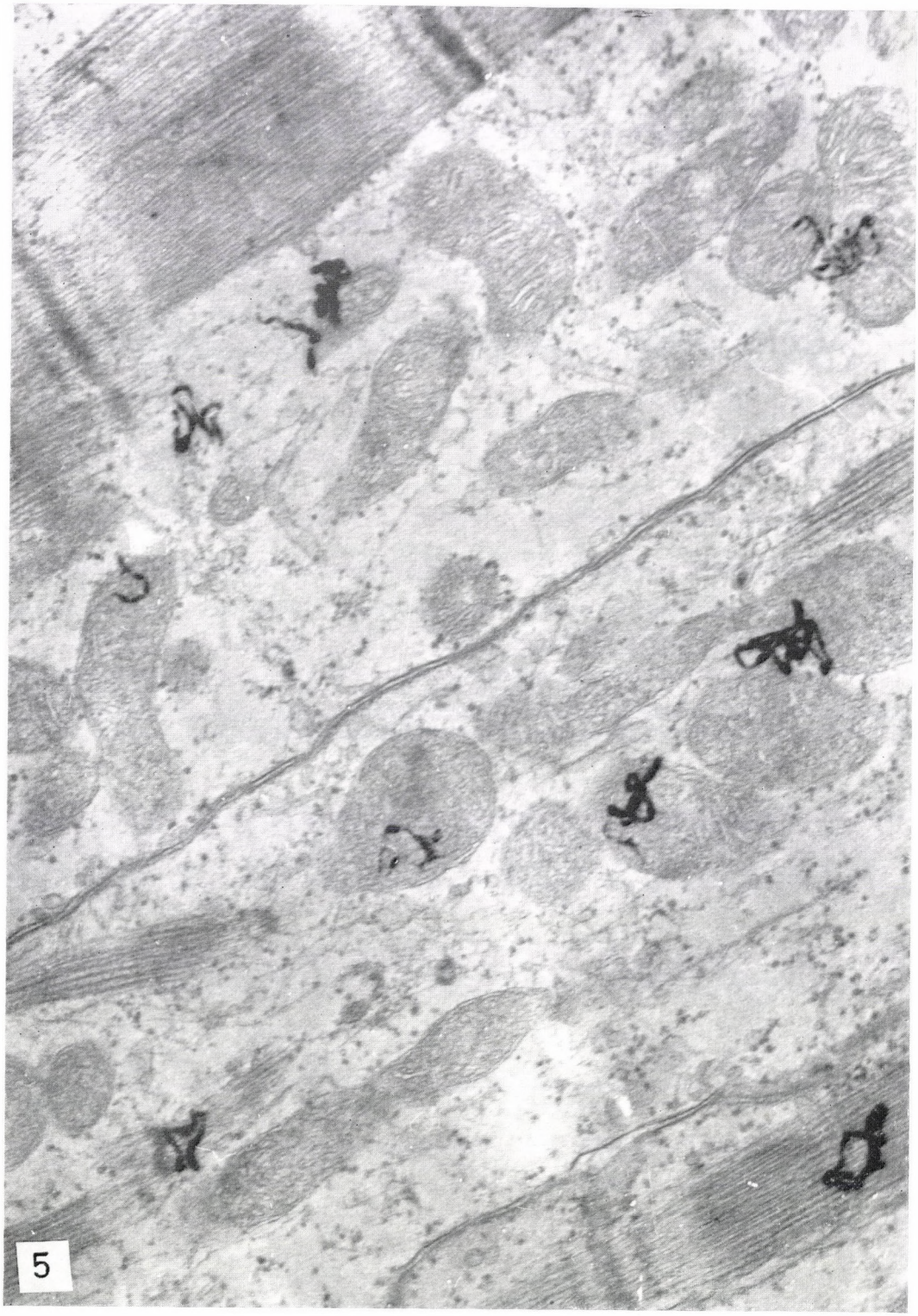

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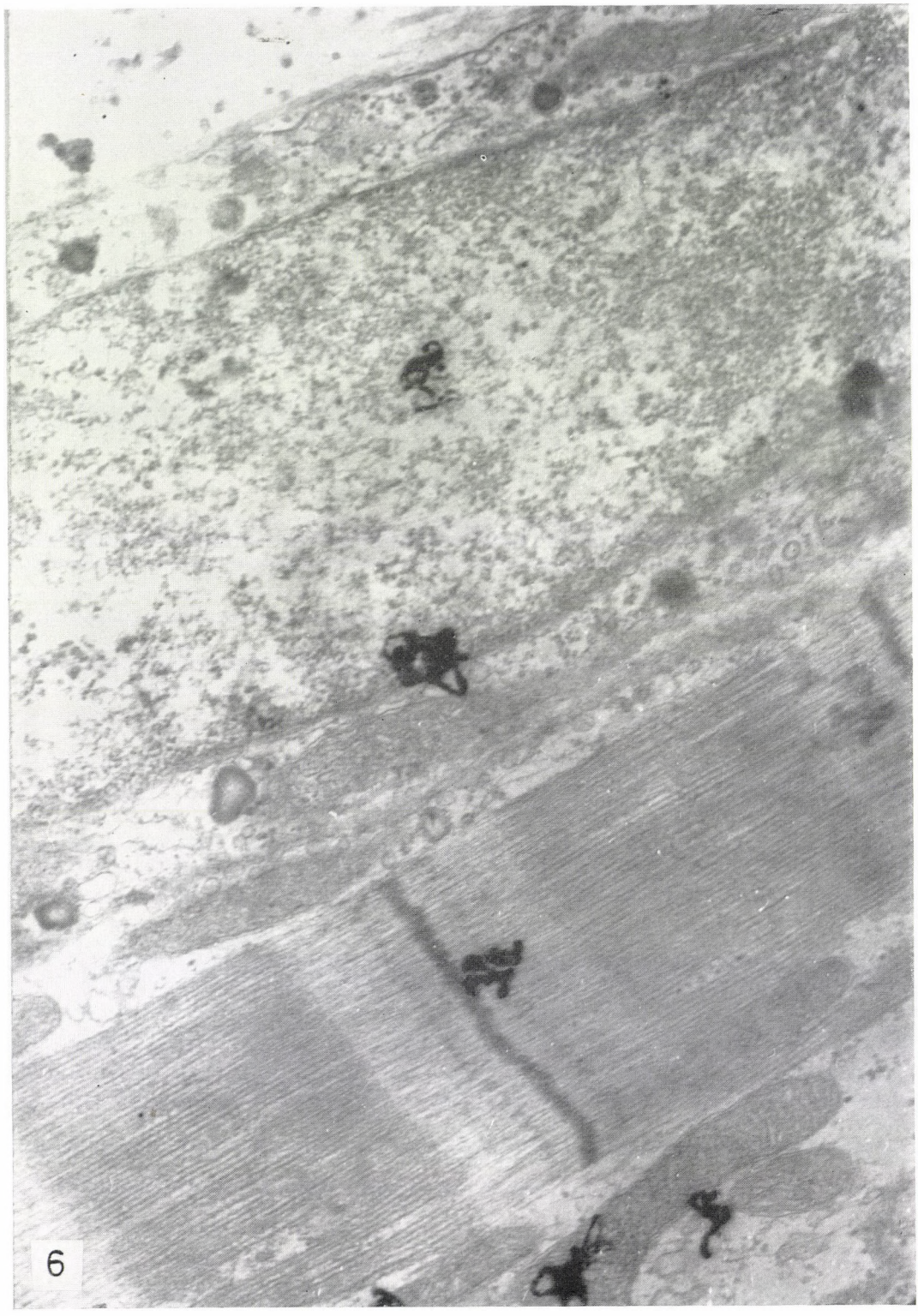
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Fig. 1. An electron microscopic autoradiogram of a longitudinal section of ventricular muscle. Magnification 20 000

Fig. 2. An electron microscopic autoradiogram of a longitudinal section of muscle of the sinus node. Magnification 20 000

Fig. 3. An electron microscopic autoradiogram of a longitudinal section of auricular muscle. Magnification 20 000

Fig. 4. Autoradiographic grains above the tubular system of sacroplasmic reticulum. Magnification 30 000

Fig. 5. Autoradiographic grains above the mitochondrium. Magnification 20 000

Fig.6. Autoradiographic grains above a nucleus of muscle cell. Magnification 30 000

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123-IODINE HEPTADECANOIC ACID (HDA) CARDIAC METABOLISM OF INACTIVE SPORTSMEN WITH NUCLEAR CARDIOLOGY, IMPACTED INTO COMPLEX CARDIOLOGICAL STUDIES

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(Received January 26, 1987)

SUMMARY

In 8 sportsmen and 8 healthy subjects of similar middle age the examination of the myocardial uptake of 123-I HDA and 201-Tl exhibited a correlation of r=0.88 using our own quantitative circumferential evaluation program. The myocardial kinetics of the 123-I HDA was assessed by different methods: 1./ according to Feinendegen et al. (1981), with complementary NaI-123 injection for the extra-myocardial background correction, 2./ according to Dudczak (1984), with bi-exponential peeling, similar to the simplified method of the van Eenige group (1984, 1985). The utilisation-elimination kinetics resulted in comparable results with correlation coefficients of r=0.80 and they were in accordance with the literature data; so this examination can be carried out without surplus NaI-123 injection, as well. The only significant difference was observed in some sportsmen who, after discontinuing the competitions, had a minimal delay of the HDA-kinetics in the septal region, where the echo-CG registered septal hypertrophy, as well.

Otherwise the data of the HDA-kinetics, of the 201-Tl uptake and wash-out redistribution were in agreement with the results of the global and regional left ventricular ejection fractions, with the ECG at rest and during ergometric stress with the mechano-CG and with the cardio-volumetry, all exhibiting overlaps in the normal range between the sportsmen and healthy controls.

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INTRODUCTION

Since on the basis of animal experiments Bing in his Harvey study in 1954 (1) declared the fatty acids as main myocardial energy fuels, great efforst were made to realize the usefulness of these experiments for the human pathology. The more sophisticated knowledge didn't alter this tendency, and the ratio of the different energy fuels under different conditions is still a matter of discussion. Evans and Gunton (1965) were the first who attempted to detect fatty acids in the human myocardium by means of radiolabel, but the real solution was presented only in 1979 by the 123-I labelling of the heptadecanoic acid (HDA) in omega-position according to the procedure of Stöcklin (1980). Vyska et al. (1979) performed not only myocardial scintigraphy, but they studied the metabolic kinetics, as well. Machulla et al. (1980) also introduced the omega-labelled p-phenyl-pentadecanoid acid, a non-deiodinating radiopharmacon, with slower kinetics than the HDA. Dudczak (1983), in Vienna, compared this new radiopharmacon with the HDA in fundamental biochemical experimental and clinical investigations; the perfusional uptakes of 201-Tl and 123-HDA were studied simultaneously. These studies were carried out by scintillation gamma camera. Meanwhile Schelbert et al. (USA research group, 1982-1983) adapted the technique of positron emission tomography (PET) with 11-C palmitate labelling. The results gathered in acute and chronic myocardial ischaemia were practically identical in the USA and Vienna. The Vienna School assumed a definite point of view in the question of extracardial, non-myocardial background correction, supported later by van Eenige et al. (1984-.1985), namely, that the complementary 2nd NaI-123 injection is not indispensable, the correction can be done by bi-exponential kinetics and with similar results.

The aim of the study

The aim of our clinical experiment was to study the myocardial metabolism of fatty acids in inactive sportsmen with cardiac hypertrophy, in the context of classical clinical investigations. As control group served heavy physical workers also consenting to the investigations. Eight formerly first class sportsmen and 8 heavy physical workers were examined. Their mean age was 36 yrs, their body surface area 2.0 m^2 in both groups and all

TABLE I

METHODS

123-IODINE MYOCARDIAL HEPTADECANOIC SCAN (whole heart and sectorial)
uptake and elimination wash out kinetics

First compartment of the v. cava sup. background corrected biexponential kinetics
Feinendegen method with 123-I Na corrected myocardial intracellular fatty acid kinetics
Vienna School method (Dudczak) with biexponential peeling method of the 2 compartment Biexp. method similar to the van Eenige and Cow. modification

201-THALLIUM SCAN (whole heart and sectorial)
uptake and wash out kinetics at rest and during ergometric exercise (redistribution)
99m-TECHNETIUM PYP-RBC VENTRICULOGRAPHY Pyrophosphat labelled red blood cell indicator
left ventricular global and regional ejection fraction (EF%) at rest and during ergometry
12-LEAD ELECTROCARDIOGRAPHY at rest and during ergometry
M-MODE ECHO-CARDIOGRAPHY at rest and during ergometry
X-RAY CARDIO-VOLUMETRY at rest
MECHANO-CARDIOGRAPHY (apex-CG with PEP/LVET)

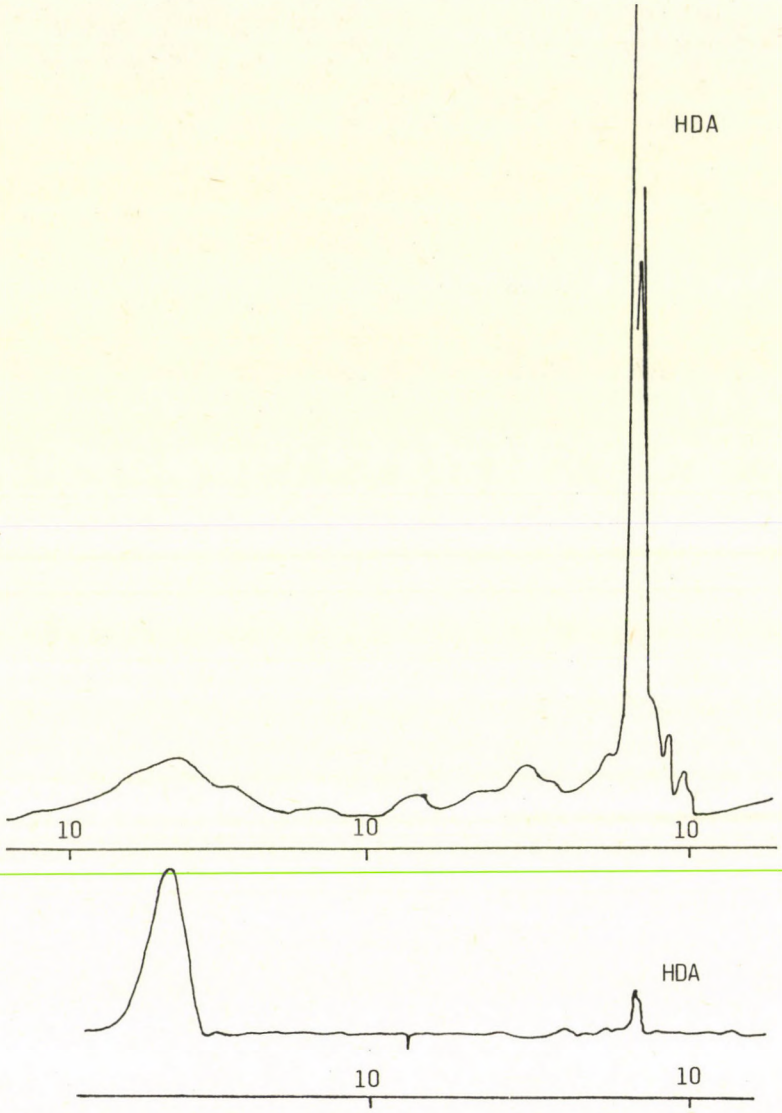

Fig. 1. Heptadecanoic acid (HDA) chromatograms:
above HDA human radiopharmacon of Squibb with weightless quantity of ^{123}JNa and mixed in bile acid and lecithin solution
below HDA compound pro anal quality of Sigma
the two characteristic peaks are in coincidence, apart from the concentration relations.

were normotensives. The cardiac volume determined by X-ray measurements was at rest 494±90 and 462±104 ml/m^2 for sportsmen and controls, respectively. The sportsmen group consisted of 2 football players, 2 tennis players, 1 basket ball player, 1 volley ball player, 1 table tennis player and 1 middle distance runner champion.

MATERIALS AND METHODS

Figure 1 shows the chromatogram of the 123-I heptadecanoic acid (HDA) Table I summarizes the methods used.

The 123-I HDA radio-pharmacon and NaI-123 of Squibb were used together for studying the myocardial uptake scan and the utilisation-elimination kinetics, according to the directives of the Feinendegen group (1981). In other cases 123-I HDA alone, was given according to the instructions of the Vienna group, led by Dudczak. For the quantitative evaluation of the regional distribution our own circumferential program and bi-exponential peeling were employed, based on the adjunctive Supersegams program of the School of Nucl. Med. of the Szeged Med. University. With time resolution of half seconds, the t-max of the uptake, the t 1/2 of the utilisation-elimination kinetics, and the ratios of the first (fast) and of the second (slow) compartments were calculated with corrections by subtracting the v. cava sup. background time-activity curves. Otherwise in cases of a 2nd NaI-123 injection the correction was performed by means of the equation of Feinendegen et al. (1981). Scans left anterior oblique from LAO-45^o were performed of the whole heart and of the septal, inferior-apical, and postero-lateral regions with parallel hole low energy collimator. The myocardial perfusion of the 201-Tl was examined globally and regionally at rest and during ergometric stress, the redistribution after 2 and 4 hours was also quantitatively evaluated. We had a special interest in the presentation of the hypertrophic right ventricle on the 201-Tl scan. The left ventricular contraction dynamics were simultaneously investigated with 99m-Tc RBC ventriculography at rest and during ergometric load and we used for the evaluation the norms of our own equipments, which have been described elsewhere (Horváth et al., 1986).

TABLE II.

123-I-HEPTADECANOIC ACID MYOCARDIAL KINETICS PARAMETERS

8 heavy physical hospital workers

			X ±SD
age	29-42 years		
BSA (body surface area)	2.0 m^2		
Myocardial time-activity curve after subtracting the v.cava sup. activity		peak time t - max	6.94 ± 1.2 min
		elimination t 1/2	
Feinendegen method			30.0 ± 7.6 min
biexponential method similar to that of van Eenige et al. but with v. cava sup. activity subtraction			27.3 ± 4.6
Vienna School method (Dudczak et al.)		t a'	10.2 ± 3.5
		t b	about 60
		t a	27.8 ± 2.6
		C A/B	1.35± 0.14

According to Feinendegen and Freundlieb

The myocardial HDA kinetics corrected by second can also be expressed by an elimination constant, NaI-123 inject the normal average being 0.026/min, the pathological one 0.022/min, with overlap between them. Converted to t 1/2 values these are 19 and 23 min, respectively.

Accumulation t 1/2 (as marker of the energy utilization)	at rest	24± 10 min
	exercise stress	22± 4
energy utilization)	test	

Normal values of Dudczak

v. cava sup. corrected monoexponential	t 1/2	22.7± 5.2 min
v. cava sup. corrected biexponential	t a'	9.5± 2.9
	t b	49.0± 19
	C_A/C_B	1.52± 0.64

Cross checks of the t 1/2 of methods

Iv. injection without background correction	Correction with second NaI-123 inj. Feinendegen-Freundlieb	Own biexponential with subtraction of the residual background after 1 hr.	Intravenous biexponential according to van Eenige et al.
49 ± 9 min	30 ± 7.6 min	23 ± 4.5 min p r a c t i c a l l y with the intracoronary	20 ± 5 min i d e n t i c a l injection of HDA

THIS CROSS CHECKS ARE IMPORTANT CONTRIBUTION TO THE STANDARDIZATION OF THE VARIOUS MEASURING TECHNIQUES OF THE 123-I HEPTADECANOIC ACID METABOLIC KINETICS OF THE HUMAN HEART

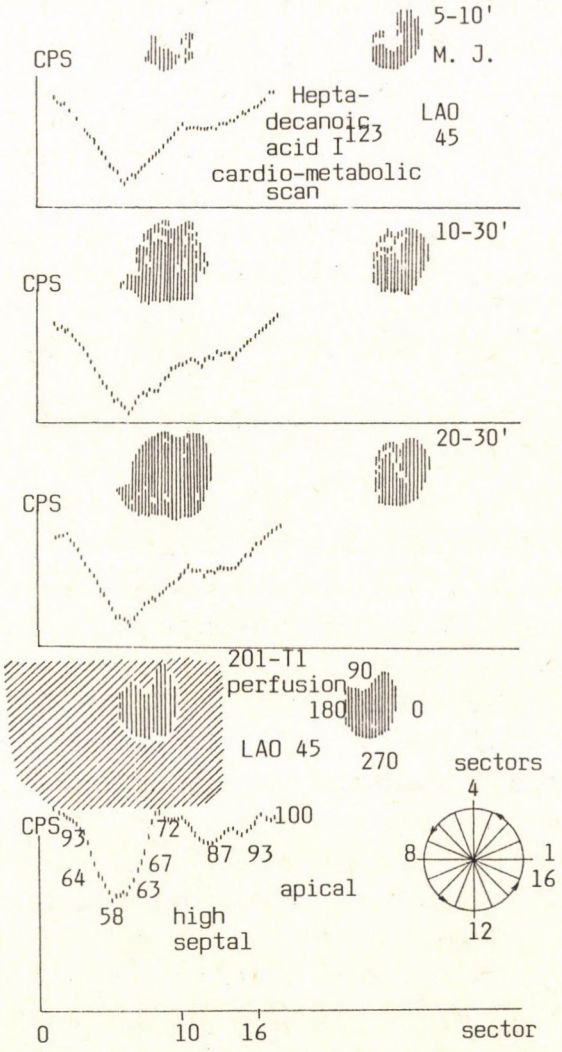

Fig. 2 Myocardial scans and circumferential profilograms of ^{201}Tl perfusion and ^{123}J-HDA uptake of one sportsman.

In Table II values of normal HDA kinetics in control heavy physical workers and, for comparison, the corresponding data of the Feinendegen and Dudczak group are shown in Table II.

The reproducibility has to be mentioned here: examined on the same subjects in repeated investigations the t 1/2 values of the first exponential were 27.7, 30.8 and 28.5 min.

Besides nuclear cardiology from the classical diagnostic arsenal we used the 12-lead ECG at rest and during ergometry, the M-mode echo-CG with Picker-80, the mechano-CG for the determination of the PEP/LVET quotient. The criteria of hypertrophy were similar to the international ones: in the case of echo-CG a septum enlargement of more than 13 mm, E-IVS[x] distance more than 12 mm and abnormal septal movement, in the case of mechano-CG the quotient of PEP/LVET over 0.37. The cardio-volumetry by X-ray, as mentioned above, was used to assess the grade of the cardiac hypertrophy.

RESULTS

It was interesting to compare the early 201-Tl scan and the 123-I HDA early uptake (Fig. 2), reflecting principally identical perfusion distributions. An high incidence of septal hypoperfusion can be demonstrated using our own circumferential program (Horváth et al., 1984).

Table III summarizes the comparison of the 201-Tl scan and of the 123-I HDA uptake (on the basis of the quantitative circumferential program), taking into consideration the type of sport, the hypertrophic an ischaemic signs on the ECG, the stress Tl-scan and the M-model echo-CG at rest.

Any anomaly on the Tl-scan of the sportsmen was observed only by a cut off criterium of 20 per cent deficit during ergometry, the redistribution was, however, in these cases normal. Qualifying on the basis of 25 per cent deficit, as it is generally accepted, the early 201-Tl perfusion of all sportsmen and all physical workers were normal. Comparing these basal

[x]E-IVS: the distance of the anterior mitral leaflet and of the interventricular septum on the K-made echocardiogram in the diastole.

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TABLE III.

123-IODINE HEPTADECANOIC ACID CARDIAC METABOLISM OF SEVERAL INACTIVE SPORTSMEN IMPACTED INTO COMPLEX CARDIOLOGICAL STUDIES

Inactive competitor sportsmen with cardiac hypertrophy	N=8	Heavy physical workers	N=8
2.0 m^2	body surface area (BSA)	2.0 m^2	
	X-ray cardio - volumetry at rest		
494±90 ml/m^2		462±104 ml/m^2	
	201-Tl myocardial exercise uptake deficit		
all normals	more than 25% hypoperfusion	all normals	
all normals	basal uptake	all normals	

basal quantitative circumferential 201-Tl and 123-I HDA scans of less than 25% deficit correlation coefficient = 0.88

Kind of sport	E C G	201-Tl exercise scan hypoperfusion less than 20%	201-Tl uptake right ventricle presentation	Basal echo-CG	Extreme cardiac hypertr.
football	LV-HT	s e p t a l	+	S+	556 ml/m^2
middle distance runner S-T depr. 1 mm		s e p t a l	+	S+	
tennis (+heavy physical work depression S-T depr. + VES		a n t e r o - s e p t a l	+	S+	
football	LV-HT	a n t e r o - s e p t a l	+	S±	
tennis		s e p t a l		S±	
volley ball	LV-HT	s e p t a l	+	S+	
table tennis		a p i c a l			
tennis					

LV-HT left ventricular hypertrophy

VES ventricular extrasystole

The septal differencies might be explained to some extent by the fact that the postero-lateral region is thicker, and thereby the high septal less is presented to the camera

quantitative 201-Tl circumferential scans of less than 20 per cent deficit with the basal 123-I HDA-scans, a correlation coefficient of r=0.88 could be assessed. In detail, the 201-Tl deficiencies exceeding 20 per cent in sportsmen were localized in 5 cases high in the septal and in 2 cases in the antero-septal region, otherwise in 4 cases the signs indicated right ventricular hypertrophy, too. It is important to point out that in the same sportsmen ECG-signs of the cardiac (left ventricular, LV) hypertrophy were also detected.

It is interesting to seek parallelism between the subclinic 201-Tl data and the basal M-mode echo-CG. The echo-signs, mentioned above occurred in 4 cases, among them in one of the football players with a cardiac volume of 556 ml/m^2 and with hypertrophic ECG-signs; in the middle distance runner who produced ST depression of 1 mm during ergometry, and in the tennis champion with antero-septal hypoperfusion, ventricular ES and ST-depression.

Figure 3 shows the HDA kinetic parameters, grouping in tripartite blocks left side of the physical workers, right of the sportsmen, in the middle their common averages presented, except the C A/B^x where the sequence, according to the meaning, is inverted.

In the t-max, in the t 1/2 of the first exponential, in the t 1/2 of the elimination according to Feinendegen, the differences are insignificant, the time data of the physical workers are, however, somewhat faster than those of the sportsmen with slower kinetics. But in some cases, it could be regionally observed t 1/2 values over the limiting 32 min, i.e. cut off discrimination.

On the basis of the correlations between the analogous kinetic parameters, and taking into account the C A/B ratios as well, we feel justified to agree with Dudczak (1984) and the van Eenige group (1984), that the kinetic values got by the two methods are comparable and conclusions may be drawn solely on the basis of C A/B data.

We examined, as positive controls, postinfarction patients and studied their I-HDA-kinetic outside the scars, in ischaemic stenotic zones, where the blood supply still resulted in some uptake.

^{x} CA/B reextrapolated intersections on the ordinate of the kinetic trends of the fast A and slow B compartments

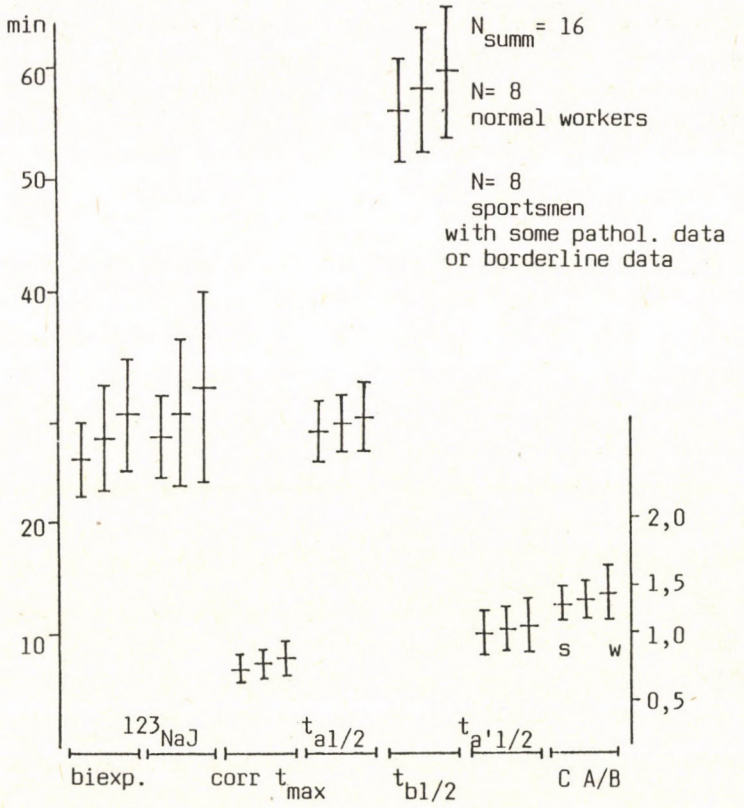

Fig. 3 123-Iodine heptadecanoic acid kinetics
^{123}J-HDA metabolic kinetic parameters of 8 sportsmen and 8 heavy physical workers: t 1/2 of the first exponential part of the curve, after VCS background substraction, t 1/2 of the elimination after Feinendegen, with the ^{123}JNa quotients and the C A/B ratios calculated from them.

Figure 4 demonstrates representative schematic diagrams about the evaluation procedure of Dudczak and the Vienna school. The peeling of the C A/B values from the two compartments results in 1.25 for the healthy subjects, in 1.27 for the inactive sportsmen, and in 0.93 for the three-vessel coronary heart disease patients.

In Figure 5 the only difference of the averages of the regional ejection fractions is demonstrated for the septal region, but this average is still within the normal range and it can be interpreted as sign of minimal septal hypokinesis in the sportsmen.

Figure 6 summarizes the test data of a first class football player. The HDA-kinetics data of the whole heart were normal by both versions of the evaluation: left the van Eenige-like method of our own, in the insert the data according to the method of the Vienna school. Although the HDA kinetics data were in the normal range, the cardiac hypertropy with 556 ml/m^2 cardio-volumetry was remarkable as was the presentation of the right ventricle on the 201-Tl scan. The stress-induced minimal septal hypoperfusional (201-Tl) and the questionable echo-signs did not point to kinetic HDA-disorders of the heart as a whole but referred to septal disorders.

Figure 7 summarizes the second model case, the data of the former middle distance runner champion. This represents a slightly delayed septal HDA elimination-utilisation kinetics, evaluated either by the principle of Feinendegen et al. or by our own bi-exponential method. The spetal t 1/2 unequivocally exceeded the limiting value of 32 min, and the t-max was also somewhat prolonged. The Tl-scan found only insignificant mild stress-induced hypo-perfusion in the anterior and the septal zones, again detectable mainly in the rear position. During the 240W ergometric stress the heart rate was 150/min and there was an ST-depression of 1 mm. The right ventricle was presented, the septum on the M-mode echo-CG enlarged and the E-IVS elonged over 12 mm.

DISCUSSION

Our starting point was that metabolic and structural disturbances have to be reckoned with in cardiac hypertrophic sportsmen, after discontinuing the competitions, even in the phase of the impairing myocardial supply. HDA-data of congestive (and hypertrophic) cardiomyopathies have been

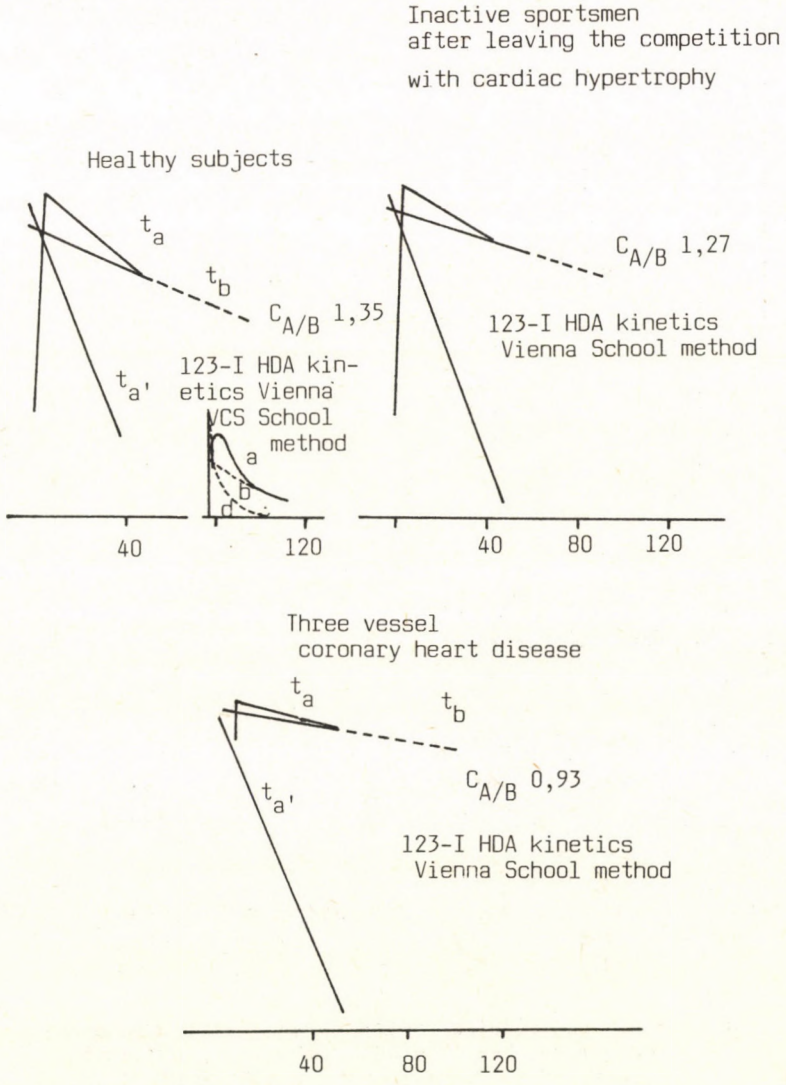

Fig. 4 Representative diagrams about the evaluation procedures of the Vienna School after Dudczak.

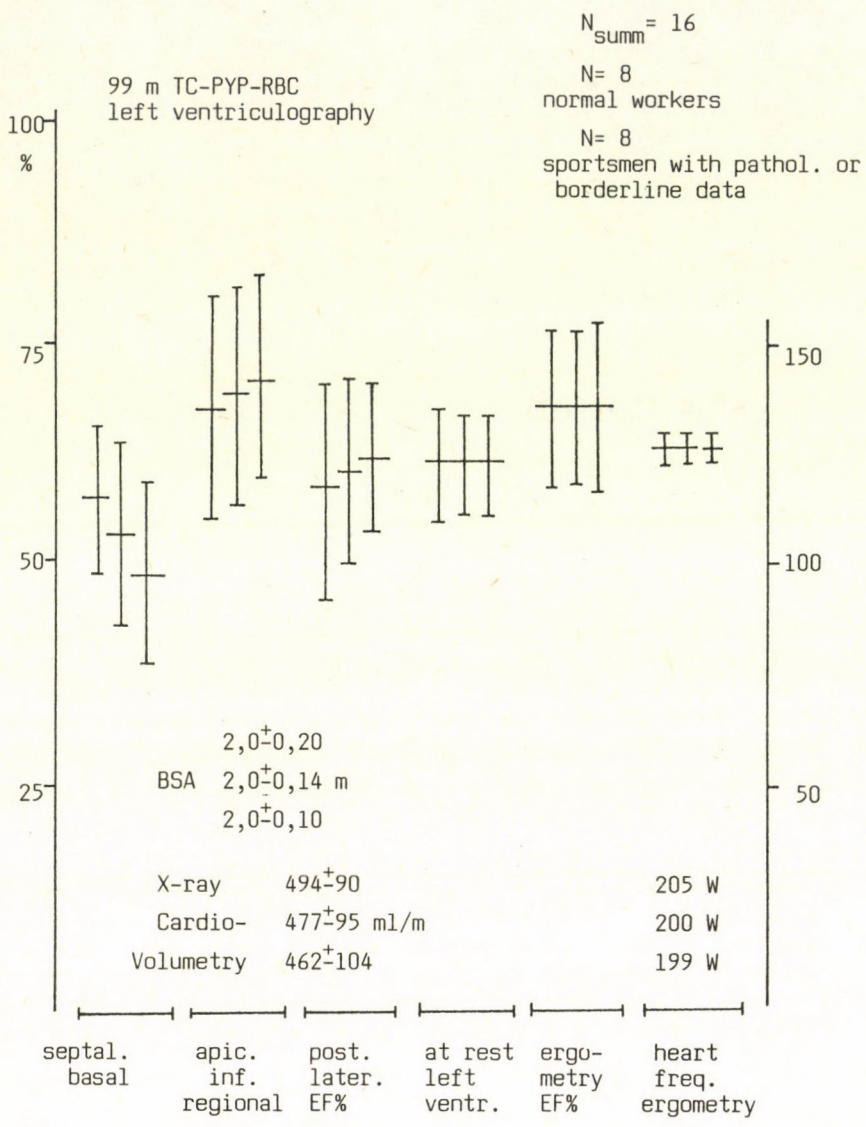

Fig. 5 Left ventricular contraction dynamics parameters determined by ^{99m}Tc RBC radionuclid ventriculography in the septal, inf. apical, post. lateral areas and the global left ventricular ejection fraction at rest and the global LV-EF reaction during stress (in average 200W ergometry and 125/min heart frequency). The difference between the values of the physical workers and sportsmen is here also insignificant.

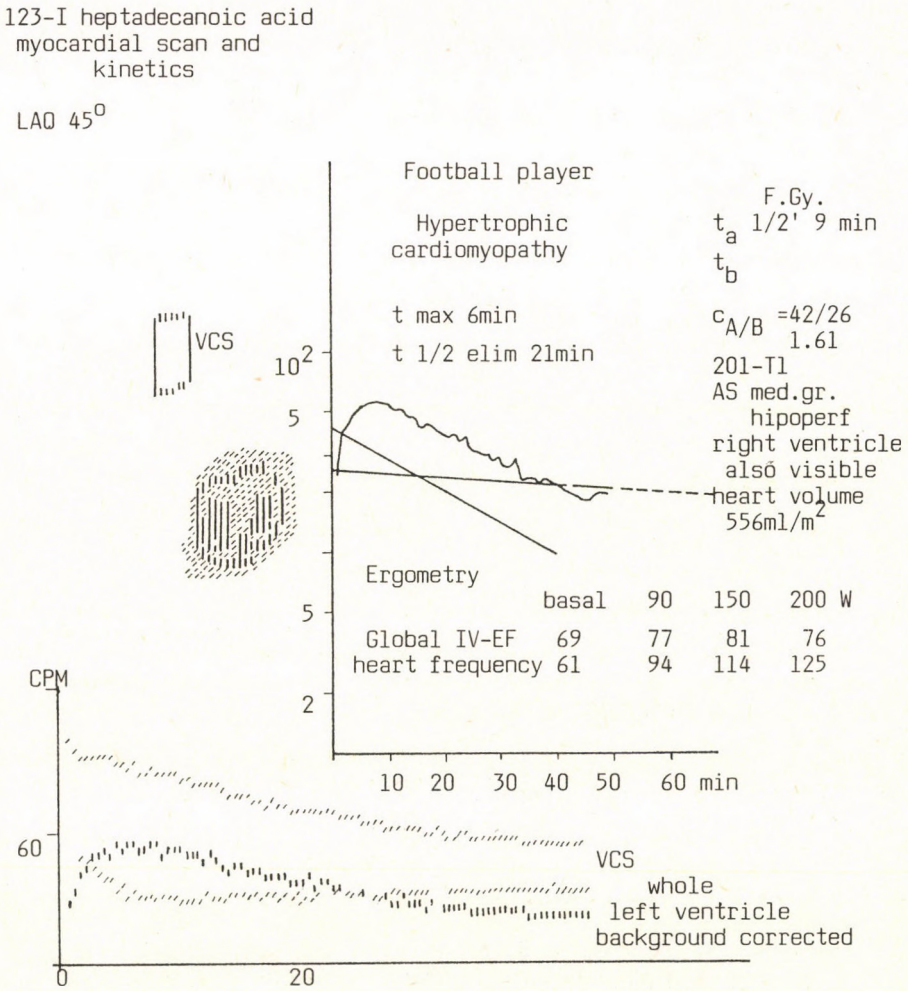

Fig. 6 Synoptic table about the examinations of the former first class football player with secondary cardiac bilateral hypertrophy (X-ray car. volum. 556 ml/m^2). The HDA kinetic of the whole heart is presented with two kinds of evaluation, but with similar t-may 6.5 min and elimination t 1/2 values 21-22 min. The C A/B 1.61 is also in the normal zone, but on the ^{201}Tl scan med. gr. ant.septal hypoperfusion has been observed. The stress LV-EF reaction was also unoquivocally normal.

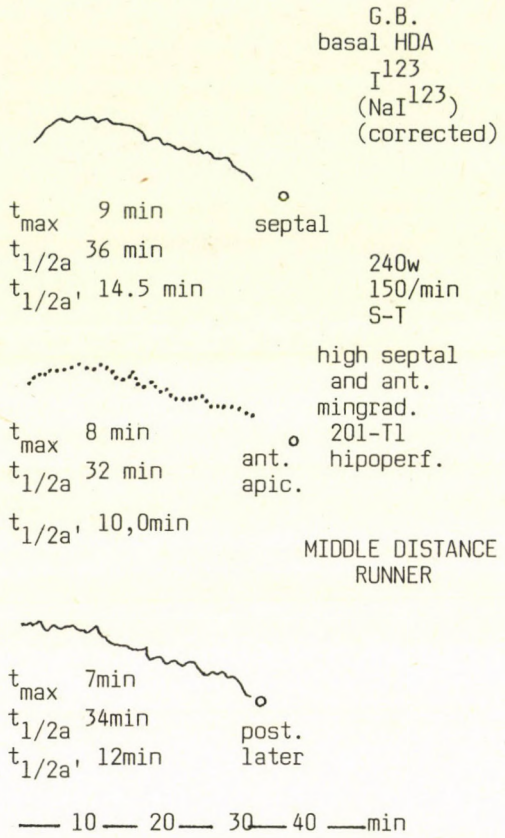

Fig. 7 HDA regional metabolic kinetic parameters of the former middle-distance runner champion, after the biexponential and ^{123}JNa background corrected evaluation. During 240W ergometric load with 150/min heart frequency high septal and min.gr. anterior hypoperfusion is visible on the ^{201}Tl scan. The regional t 1/2 a' is only in the septal region over the cut off limit of 32 min and this is an accordance with the peeling t 1/2 parameters of the Vienna School.

published by Höck et al. (1983) and by Fridrich et al. (1984).

Though our global myocardial HDA-kinetics parameters of sportsmen are still in the normal range the septal anomaly verified by different investigating methods deserves attention. The geometry given for detection, the poor spatial resolution in the rear high septal area can not be neglected, but the septal lesion of the hypertrophic heart is not at all an unknown phenomenon (Pachinger et al., 1980). Dudczak in his monography "Cardiomyopathies" also published a figure, in which the HDA uptake diminished in the upper septal region and the right heart was also clearly presented. There are similar spares data in Table 5 of Fridrich et al. (1984). The regional septal hypo-perfusion and HDA-kinetics disorder could not be of serious character in our inactive sportsmen because they were not completed with bundle branch block.

Végh, Törő and Sereg (1986) published frequent occurrence of cardiac hypertrophy in sportsmen during one year follow-up by 2-D echo-CG. According to Wenger and Gilbert (1974) the increased ratio of the heart/body weight disappears when the champions discontinue the competitions. Therefore the functional study of the inactive sportsmen is interesting. How much any change is the consequence of an early pathological process, one could decide only - as Lie et al. (1985) did - on the basis of the tendency observed during follow up. We should like to collect further experience in the future. The pathophysiological interpretation of the observed kinetics is a fairly complicated problem, because many anabolic and catabolic by products occur in the course of the 123-I HDA metabolism in the myocardium. They are influences by many, sometimes unheeded circumstances as the fasting state, excitement, glucose-insulin effect, albumin/free fatty acid ratio, different drug effects (e.g. beta-blockers), alcohol and from the pharmacological side also the carrier medium, e.g. bile-acid-lecithin. It must be borne in mind that the fate of the radioactive marker in the case of the 123-I HDA too, is not necessarily identical with that of the whole labelled molecule. Visser et al. (1985) followed on the relationship between the HDA-elimination and the oxidative metabolism of excised dog-hearts at different time-intervals after the injection. The HDA is taken into the lipids or it is dehalogenized so, the elimination rate can be determined mainly by the wash-out of the free iodine. A change of the elimination might be produced either by the

diffusion of the free iodine or by the shift of the ratio of the free iodine and lipid iodine. We followed also the appearance of the free iodine in the thyroid gland and somewhat later the enrichment in the hepatic lipid pool (which, with time, reduces the heart/liver activity ratio). Sobel (1985) in his comprehensive lecture based on 11-C palmitate studies with PET called to the fact attention that in the detected zone there are also other elements than myocardial cells thus, the relation of the oxidized fatty acid quantity/min to the real myocardial mass is stochiometrically unknown. Finally, one can get the gist of the myocardial free fatty acid (FFA) kinetic cycle from the simplified scheme of Feinendegen et al. (1981), completed by the data about the distribution in the different lipid pools during the cycle, published by Schelbert et al. (1982-83). The FFA in the cytosol enters into the coenzyme A-system by means of ATP and through the carnitine shuttle, more than 50 per cent, arrives to the mitochondria, where they are to be utilized. In the mitochondria, being linked again with coenzyme-A, they will be converted through beta-oxidation, and finish off as CO_2 in the terminal stage of the respiratory chain. So, the 11-C of the palmitate gets from the oxidized FFA-s into the CO_2 end-product. In the intermediary acyl-coenzyme phase acyl-derivatives are produced, some remaining in the myocardium, others of low molecules being excreted.

For conclusion it must be pointed out that for human metabolic study the natural palmitate is the best one with 11-C label, but other long chain derivatives can also be used. The kinetics of the short chain fatty acids are faster than that of the physiological C-16 and C-18 derivatives, while the kinetics of the 123-I p-phenylpentadecanoic acid is slower than that of the similarly labelled heptadecanoic acid. The beta-oxidation of the fatty acids of even numbers produce only acetic acid, while that of the uneven ones produce both acetic acid and propionic acid.

For the covalent binding of the radio-iodine markers the omega-position is essential. The methyl-branched fatty acids are inadequate for the metabolic studies, they are used only for the uptake scan in the myocardium.

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BOOK REVIEW

BASIC ENZYME KINETICS by T. Keleti (translated by M. Kramer), Akadémiai Kiadó, Budapest, Hungary, 1986, 421 + XVI pages, 75 figures, ISBN 963 05 4090 8.

Enzyme kinetics is an everyday means for many of those who are working in the field of biology and who are interested in learning the rate and character of different changes occurring in living organisms. Therefore, it is indispensable not only for biochemists but also for theoreticians and those working in medicine, agriculture or pharmaceutical research and industry.

No wonder that quite a few monographies have been published during the past decade dealing with enzymes and their kinetic properties. These discuss the problems, evidently, according to the individual approach and experiences of their authors.

Dr Keleti's aim is, as he makes it clear in the preface to the volume, "to help the biochemist at the bench in the laboratory". It is a good intention, so much the more, since the rapid development of equipments, the all-round utilization of computers, various sophisticated equipments and an increase in the number of commercially available compounds produce new results almost day by day, altering traditional procedures and forcing scientists to learn permanently.

The book is the work of a competent scientist whose purpose is to help those who are less at home in the field but, for one reason or another, they have to deal with its problems.

The volume is divided into three parts. The first section summarizes the fundamental laws of thermodynamics, the knowledge of which is essential for those being engaged in kinetics. The second chapter, under the title of Chemical Kinetics, analyses the general principles of kinetics, such as reaction rate, molecularity and order, reversibility, homogenous or heterogenous catalysis, the effect of medium and so on. Besides, it provides a good systematization of the most generally applied measuring procedures and calculations with which kinetic para-

meters can be determined. The third chapter covers about two-third of the book and is the most detailed discussion of enzyme kinetics itself. In the first place, it treats the classical Michaelis-Menten kinetics and analyses the meaning and calculation of the different constants characteristic of enzyme-substrate interactions. The author deals with the problems of the manifold types of inhibitions as well. The best part of the chapter is the critical examination of the models concerning the possibility of enzyme regulation and its mechanism.

The reader will surely make use of the calculation methods compiled in the Appendix and the selected bibliography that can be found at the end of the volume.

As the book provides useful informations on enzyme kinetics in the broadest sense, it gives a good help for those working in this field.

P. Elődi

CHROMATOGRAPHY

These Chromatography conferences are part of the series of American—Eastern European Symposia on Liquid Chromatography initiated in 1981. The interest in these symposia has been increasing ever since due to the high scholarly standard. The conferences are regularly attended by chromatographers from up to twenty countries including the USA, the USSR, Sweden, Canada, the GDR, Italy and France.

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Proceedings of the Budapest Chromatography Conference
June, 1985, Budapest, Hungary
Edited by H. Kalász and L. S. Ettre

1987. 712 pages. 17×25 cm. Hardcover $66,00
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The scope of the scientific contributions range from theory to practice and from gas-liquid chromatography, through GC-MS, to high performance liquid chromatography. A considerable portion of the posters and lectures deal with the biological and medical application of liquid chromatography. The most interesting results and the advances in this field have always been on the program of this symposia series, thereby this new conference yielded such interesting presentation as HPLC of cloned insuline, analyses of free amino acids in a quarter of an hour, determination of various substances from serum or urine, HPLC of ecdy-steroids, isotachophoresis of oligopeptides, etc.

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Proceedings of the Advances in Liquid Chromatography
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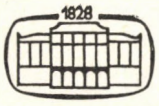

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THE CHARISMATIC TEACHER AT SZEGED: ALBERT SZENT-GYÖRGYI*

F. Bruno Straub

Hungarian Academy of Science Budapest, V., Roosevelt tér 9.

(Received June 14, 1987)

My good fortune brought me in contact with Albert Szent-Györgyi at the beginning of 1932 and my life became closely associated with his laboratory for fifteen years. The list of succesful scientists who spent a number of years there at time, who became well-known biochemists on their own is very impressive. Some are already lost, like Kálmán Laki, Mihály Gerendás, Jenő Ernst, Béla Gözsy, László Vargha, others are still with us, like Ilona Banga, Sándor Szalay, Joseph Svirbely, W.F.H.M. Mommaerts, Tamás Erdős, László Loránd, John Gergely.

The Editor of this Journal asked my contribution to this memorial issue and I thought best to recall the impression of the youngster I was coming under the influence of a great man. There are only a few who can today recall the events of 55 years ago. I was 18 at the time, having started medical studies at Szeged University, with the intention of becoming a practitioner, to do honest work as was tradition in our family. I did not know much about the world or about science, even less about chemistry. This was all mostly obscured by a strict education at the high school level. I was encouraged to believe not do doubt things.

* *In memoriam Albert Szent-Györgyi*

Akadémiai Kiadó, Budapest

Then all of a sudden a whirlwind has swept me off my feet: the personality of Albert Szent-Györgyi convinced me to understand and adopt different values towards the world, towards science, towards research. I believe most of the younger generation joining his laboratory experienced the same, which I am trying to describe.

The first impact was the way he gave his lectures. At the lectures I heard in Physics, Anatomy, Histology superior but mostly dull people were giving us data to learn, told us laws we had to be able to memorize. Then came the chemistry class and Albert Szent-Györgyi came in telling us in simple words what the problems are, what are the presently known principles, what is the beauty e.g. in stoichiometric reactions, why the concept of pH is helping us to understand the phenomena of life. Everything became clear and easy to understand, every new idea seemed to be further developed. We were given to understand that it is not the detail, but the basic idea which had to be grasped. We realized the deep effort and enthusiasm which the search for konowledge and understanding was radiating form our teacher. At the time in the early thirties, there were no textbooks from which one could learn the subject to pass at the examination. And the first examination turned out to be a dialogue on what we heard from Albert during the semester.

It was a bit of a surprise and a shock when Szent-Györgyi asked me whether I would like to work in his laboratory. The surprise was understandable, the shock was due to the fact I never thought of changing my chosen course, never thought of doing chemistry. However, after a few days I became convinced

that to work in the laboratory led by such a great scientist would be for my benefit. After about two years Szent-Györgyi proposed to me that I should rather leave my medical studies and learn instead some chemistry if I want to stay in biochemical research. I did not hesitate and did so. My family was somewhat skeptical, because in the early thirties in Hungary a physician was able to make a good living but nobody ever heard about a man having a Ph. D. in chemistry ever making a carreer. But I took a chance and my decision is due to another characteristic of Albert Szent-Györgyi.

What did I see, working in his laboratory? First of all an intensity of life and work. The Prof (as Albert was called) never lost a chance of discussing experiments, new ideas, music, politics, art and sports. And he had the natural gift of being able to talk and discuss problems with a young unexperienced man like myself, as if we were on an equal footing.

Moreover, already at this early time I realized that he definitely liked to be contradicted in a discussion. It was, I realized later, his greatest asset that thinking intensively on a problem, he wanted to hear what one can say <u>against</u> the idea. This phantastically enjoyable spiritual atmosphere seemed to me worth any sacrifice. The whole atmosphere emanated from him, and I took his advice.

From an apprentice I proceeded to become a scientific associate and produced papers in which I was able to add something to an idea which Albert suggested. He was generoues in giving credit to his associates. The laboratory worked mostly on the basis of a team-work. At the end of the year Albert

asked everybody to write up what they did. Then he took these papers, rewrote them in a more understandable way. He himself wrote a summary of all these papers and described what he observed and worked out with his own hands. Then he added under the name of one or two of his coworkers the details, not putting his name on it. It was a happy family.

A last remark of mine refers to a characteristic of Prof, evident from what I wrote about the publications. It was obvious for anybody who knew him that there was a very strong drive in him: to understand more about the essence of life. He often came into the laboratory excited with a new idea, saying: now I think I understand life. He easily attracted students who worked with him, he was always full with many different ideas and was able to supply any of his new students with a promising idea. Yet he never wanted to exploit any of his students, there was no selfishness in his doings. He helped many of us to obtain fellowships abroad. And - after a few years - he did not mind, if we, his students tried to strike out pursuing a research line chosen by ourselves and broke away from his group.

Thus Drs Banga, Laki, Vargha, Szalay and myself were later working in different fields, with different styles and outlook. But basically what we achieved has come about based on what we learned from the late Albert Szent-Györgyi, the Prof.

Thus in the late thirties several fairly independent lines of research were pursued by some of us. Then Prof became enthusiastic about a new idea. He saw the paper of Engelhardt and Lyubimova in "Nature" and realized the importance of the interaction between myosin and ATP. After a few preliminary observations leading to the discovery of myosin A and myosin B

he suggested a teamwork, joining our forces again on a central problem. For all of us who participated in the "Studies from the Institute of Medical Chemistry, University of Szeged" during the threatening ongoing of World War II, it was a glorious time of enjoying the spirit of discovery. Prof, the Nobel prize laureate who led this newly recreated group, was the same charismatic leader during these wartime years, as he was when I first met him. Plenty new ideas were discussed, and his attitude remained the same as before: questions have to be asked from Nature, new ideas should be discussed. We felt the joy of intellectual achievement and this has given us enough moral strength, so that (with luck) we survived the last year of the war sweeping over our country, scattering colleagues. When peace came we were reaching different new posts, but all of us tried to carry on the spirit of our teacher.

In the fall of this year a symposium will celebrate the 50th anniversary of Albert Szent-Györgyi having been awarded the Nobel-prize and the medical University at Szeged will be named after him. He will be remembered in many ways.

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THOUGHTS ON THE LATER CAREER OF ALBERT SZENT-GYORGYI*

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I did not know Albert Szent-Gyorgyi well. We were a generation apart and I did not work in his laboratory. However, for almost forty years I observed his activities each summer in and around the Marine Biological Laboratory at Woods Hole and enjoyed his zest for science and living. I admired his past achievements as a master biochemist and listened to his joyous dramatic lectures. He had peopled the United States with bright young Hungarian biochemists, many of whom are my friends, and they and I were grateful for the pleasure of knowing a heroic scientist in our own time.

He had occasionally listened to my reports of progress on the biochemistry of virus multiplication and on the effects of unusual medicinal nucleosides. We nodded and smiled in passing, but we remained a generation apart. Biochemistry and biological science generally were pulling away from him. Some of his younger friends offered to help him in some institutional problems, but he had decided not to change his way of doing science and there was little that could be done. Nevertheless his professional problems towards the end of his career are of general interest and should be addressed.

In 1963, he wrote an autobiographical essay entitled "Lost in the Twentieth Century." Containing a paean of praise for science and its moral inquiring spirit and achievements, he directed a plea to fellow

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scientists to struggle for peace and prosperity, to create "a new form of life, the wealth and beauty of which cannot be pictured today by the keenest imagination."

At that time, he had lived in the United States for 15 years and had built a functional laboratory as part of a prestigious institution in the lovely seashore town, Woods Hole, in which he lived. In that new laboratory, he had discovered the interesting properties of glycerinated muscle, and the glycerination process proved to be of widespread interest and use. Many of his students and assistants from Hungary had settled in productive positions in America. The knowledge he and his colleagues in Hungary had developed on actomyosin had been transferred to the United States, and his institution, the Marine Biological Laboratory, had become a center for research on muscle and discussion of the new results.

Summers at Woods Hole were an exciting scientific and social experience, in which he participated actively. Many figures of his earlier scientific life had turned up on occasion, including Otto Warburg, Carl Neuberg, Otto Meyerhof, and Otto Loewi. A biochemist-physiologist member of his generation, E.S. Guzman-Barron led a laboratory and lecture course in Physiology, to which Albert contributed, with many other distinguished scientists. Indeed, when this course was converted at five-year intervals to contemporary versions of the discipline, muscle remained a key segment. The "Prof's" lecture was popular and retained in the teaching sequence. With this transition to modernizing, also, came the emerging younger scientists who were appropriately interested in Albert's interests and concerns and applauded his vigorous way of life.

The vitality of this summer activity also reflected the state of American science, which was well supported and growing in leaps and bounds. The American leadership of this international science was a consequence of World War II, and a result of the destruction of much of

European civilization. But it also was due to the escape of many European scientists to America and to their contributions in research and help in training young American biochemists. I myself came from such a training center, the Department of Biochemistry at Columbia University, whose Faculty during the period of my studies included R. Schoenheimer, E. Brand, E. Chargaff, and K. Meyer. The language of science had become English, and the enormous flood of new results expressed mainly in this language poured into the superb Library of the MBL which now harbored Szent-Gyorgyi and his small group.

Nevertheless there was a fly in the ointment. Or there were flies. He had been a European and a Hungarian, and it is impossible to describe the attachments of a displaced person to his roots, to his former home, friends, familiar sights, language. I will not attempt to describe theoretical longings, with which I am not familiar, but which exist in, indeed permeate, every stratum of American society containing so many immigrants and exiles from chaotic societies all over the world. Szent-Gyorgyi had discovered also that the Promised Land was not merely a given good, but needed some input to preserve its Promise. He had resisted Hitler and quarreled with the Russians, only to be challenged by McCarthy in this new home, his presumed asylum. He was frustrated "to see mankind on the brink of extinction," "to have spent so much life and energy in vain." Nevertheless it will be remembered that he never ceased to exhort his colleagues, his neighbors, and the youth to express their democratic will.

Although unstated by him, there had also been very real changes in the structure of science, changes which had already affected his ability to make discoveries and which could only increase his difficulties in that activity. Indeed he had referred to undefined "personal limitations," but he might also have noted changes in the structure and institutions of

biomedical science, which were to affect his future work.

Szent-Gyorgyi made his important discoveries at a very early stage in the evolution of biochemistry. Although Buchner's discovery in 1897 of glycolysis in a yeast extract had established the perspective of separating cell components and analyzing the metabolic activity of purified fractions, for some time washed minced tissues remained as major biological materials for the study of catalytic properties. Only 27 years after Buchner, i.e., in only 1924, Szent-Gyorgyi had used washed muscle to demonstrate the activation of both hydrogen and oxygen in cellular oxidation. A few years later, in 1928, using effective techniques now considered primitive, he found a factor in adrenal cortex and inplants which could serve as a catalytic hydrogen carrier in selected reducing systems. This eventually proved to be ascorbic acid or vitamin C. It is instructive to note that, despite the growth of biochemical skills and human activity, many aspects of the functions and metabolic relations of ascorbic acid have not yet been clarified. His important work in 1934 to 1937 on the four-carbon dicarboxylic acid cycle employed rapidly respiring minced pigeon-breast muscle. Although he elected in 1941 to work on a muscle protein called myosin, and, with his collaborators, discovered the presence of two proteins, actin and myosin, in the functional complex of actomyosin, his research career demonstrates that cell fractionation and work with purified enzymes and proteins was not the approach he deemed most comfortable or most promising.

We note that he did not use isotopic methods in the exploration of metabolism, or explore changes in the actomyosin structure by the sophisticated physical methods introduced in the 1920's and 1930's. Viscosimetry was available in war-torn Hungary, but how does a biochemist expert in the use of minced muscle switch late in life to the most modern gear? In short, Szent-Gyorgyi, like every other scientist trained and

experienced in one era, had problems with the new theory of related disciplines and with the tools of a subsequent period. How do most of us do it, if we do it at all?

In the 1930's major laboratories had concentrated on the fractionation of cell extracts and the purification of proteins and enzymes, even of viruses. Powerful physical tools, ultracentrifuges, apparatus for electrophoresis, had improved the characterization and, in some cases even the isolation of macro molecules. Electron microscopy and X-ray crystallography were adjuncts to the difficult problems of describing proteins in isolation and in situ. Enzymology had become a fine art for the mathematically inclined students of catalysis and in its cruder aspects was essential in the analysis of reaction sequences. Isotopic methods had enormously expanded our metabolic catalogue and knowledge of catalytic mechanism. Biochemistry, in its reductionist aspects, had become the work of specialists, and the contributions of all of these had become necessary to understand the integration of the separate bits. These directions of the discipline had become evident even before the addition of the developmental disciplines in the evolving construction of the superdiscipline of molecular biology.

Szent-Gyorgyi thought (hoped?) that the new knowledge on the plethora of small metabolites and the ungainly macromolecular cellular components might prove to be too complex to account for the functionality of living tissue, as well as of the origin of cells. The whole system might possess simpler mechanisms to permit, to support, biological activity, might possess short-cuts to bypass the stuff of thousand-page textbooks, the long lists of intermediary metabolic reactions. If such a primitive simplicity had existed and had been retained Szent-Gyorgyi himself could bypass this complexity and stride like a giant past the epiphenomena described in decades of accumulated trivia. I have seen other men in

physics and biology searching for new rules with which to link these disciplines, expecting (hoping?) to bypass the awesome complexity of growing biochemical knowledge. Obviously this search has failed. After World War II, physicists came in flocks to biology to demonstrate the power of their discipline, their magical skills. In virology at least, a field with which I am familiar, almost all of these prideful searchers were silenced by primitive mouth-filling pipettes. The search for short-cuts is always with us, and Szent-Gyorgyi needed one to solve the problems of his "personal limitations."

To bypass the enormous range of substances present in the cell and their numerous reactions, observed in extracts, Szent-Gyorgyi asked if essential electron transfers had not found shorter routes. Indeed ascorbic acid, in reaction with nicotinamide and quinones, has been found to participate in such transfers, and could be isolated in "charge-transfer" complexes. Such phenomena, if they existed, were detectable in the electron spin resonance (ESR) of the complex, and he and his young collaborators sought such evidence in one of the first instruments used in this country to measure ESR. His ideas elicited much discussion among biophysicists and a certain amount of work. However, no major discoveries have been made or exploited, and the postulated direct path of Szent-Gyorgyi has not been placed in the textbooks containing his many other achievements. I have myself made a solid yellow complex of ascorbic acid and nicotinamide, all too readily dissociable in water, and have wondered about its possible physiological significance.

All maturing and mature biochemists who have made even a single discovery, face the problem of having more to do than he or she possibly can handle himself. To solve the problem of an expanding program of work in the United States he has technical assistants, students and postdoctoral fellows. He develops a division of labor, which in modern

times at least soon drives him to his desk, examining data, writing papers, and justifying and applying for financial support. The development of a research team, made possible by the prosperity of the country and his own success, inevitably separates him, at a greater or lesser rate, from the bench at which the amazing tissue, cell or substance is being examined. Very few are able to stay as close to the work as they once did. Szent-Gyorgyi rejected this solution and had decided to organize his laboratory, his working space and working life to avoid the transformation of scientist to manager. Nevertheless this very laudable decision contributed to his falling behind.

His "modest" course was possible with meager funds, possible until the funds were inadequate to maintain his self-limited needs. By this time, by 1963, many private foundations in support of research had closed this aspect of their efforts, unwilling to compete with the much larger funds made available by the United States government. In its initially enlightened policy, the government began to support Albert's work and that of many others. As requests for government support of basic research proliferated, the bureaucracy developed an enlightened theory and system of making awards in which the scientists themselves assessed and screened grant applications. The form of grant application became more and more elaborate. Young unknowns had to convince the review panels of their worth. Szent-Gyorgyi, whose discoveries had achieved world-wide recognition and honor, was now in a race for funds with worthy young applicants eager to succeed and willing to accept tasks Albert had rejected. He was not competing with these eager aspirants who for the most part were extending the past and their own recent work, to seek new and previously unsuspected biologically significant phenomena. Had not his previous record of discovery entitled him to the unextravagant support he needed?

Albert no longer fit within the categories, the rules established by government agencies. Actually few members of the review panels appreciated an elderly gentleman, who refused to abide by the rules within which they had to function. He was over seventy, why didn't he quit just as did almost everyone at that age? Why didn't he work in a field we could understand, i.e., a known field? Why didn't he publish his work in the way the other applicants had to? To make a long story short, the combination of unappreciative scientists and a fearsome bureaucracy did not support his work. After a series of wearing rejections, he turned to a special agency which helped him almost until his death.

Is there a moral from all this, are there lessons to be learned? They might be stated as follows. Young biochemists, despite an "advanced" education of a certain kind, can be ignorant and frequently unkind. Older biochemists, despite status and position, have generally adopted and accepted the standards of the society in which they have succeeded; they do not often support their older colleagues in need. Historic elderly surviving figures do not receive special consideration, nor should they expect to. The bureaucracy is predictable; rules are rules.

Albert Szent-Gyorgyi lived an extraordinary life. His work and career were like the bright burning breathing candle, whose description by Seguin and Lavoisier in 1789 illuminated biochemistry at the very beginning of our discipline. Towards the end the bright flame of his science flickered, and was extinguished as his substance was consumed. We did not know how to help him as he grew old, nor did we try to do so sufficiently. The different generations living side by side in our society seem to be living on different planets.

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EFFECTS OF CAFFEINE ON MONOAMINERGIC SYSTEMS IN MOUSE BRAIN *

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Effects of caffeine on monoamine systems in the mouse brain were studied in three lines of experiments. First, concentrations of 10^{-7} M to 10^{-2} M of caffeine were tested for their potency in inhibiting the carriers involved in the neuronal uptake of dopamine, norepinephrine, and serotonin. The IC_{50} of caffeine in inhibiting the first two carriers was approximately 10^{-2} M, and that in inhibiting the serotonin was 2×10^{-3} M. Second, concentrations of 10^{-5} M to 10^{-3} M of caffeine were tested for their potency in affecting the in vitro KCl-induced release of [^{3}H]dopamine from dopamine terminals in the striatum and from norepinephrine terminals in the hypothalamus, and the release of [^{3}H]serotonin from serotonin terminals in the striatum. Little or no effect was observed. Third, caffeine was administered for 3 weeks to mice via their drinking water at 73, 123, and 162 mg/kg per day. No changes were found in their D_2-dopaminergic, 5-HT_2-serotonergic, or α_1-adrenergic receptors in the striatum or cerebral cortex as compared with animals on normal drinking water. All these neurochemical results are consonant with the interpretation of behavioral studies suggesting that caffeine is an only mildly stimulatory drug that should not be grouped with other psychostimulant drugs such as amphetamine and cocaine that do affect monoamine systems in the brain.

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INTRODUCTION

Indirect evidence suggests that caffeine affects the release of monoamines from nerve terminals in the brain. For instance, when norepinephrine synthesis is blocked by α-methyl-p-tyrosine in the rat, caffeine reduces the concentration of norepinephrine in the brain stem, and following blockade of monoamine oxidase by pargyline, caffeine accelerates the elevation of brain stem norepinephrine (1). Similar evidence suggests that caffeine inhibits the release of dopamine (2) and of serotonin (2,3) in rat brain. The observation that many effects of caffeine are opposite to those of adenosine has led to the hypothesis that caffeine blocks adenosine receptors (4), thereby disinhibiting the effect of adenosine in reducing the release of monoamine (5,6) and amino acid (7,8) neurotransmitters. The resulting increase in the release of dopamine and serotonin is not consonant with the above interpretation of the in vivo experiments with blockers of monoamine synthesis or degradation. Xanthines have also been reported to increase the amount of serotonin in the brain, concomitantly with a rise in brain tryptophan, the precursor for serotonin (9). The latter phenomenon has been linked with a) a lipolytic effect of xanthines, causing an increase in plasma unesterified fatty acid, which in turn frees plasma tryptophan bound to albumin so that it becomes available to the brain (9); and b) an increased effectiveness of tryptophan uptake by the brain caused by increased insulin secretion (10) and/or by an enhanced ratio of tryptophan to the sum of neutral amino acids competing for the same uptake systems (11,12).

In the present work two approaches were taken to study the possible effect of caffeine on the synaptic availability of monoamine neurotransmitters. First, the potency of caffeine was studied in inhibiting

in vitro the carriers for neuronal uptake of dopamine, norepinephrine, and serotonin, and in modulating the in vitro release of these monoamines from nerve terminal preparations from mouse brain. Second, mice were treated chronically with caffeine, and monoamine receptors in their brains were assayed for possible changes. It was reasoned that sustained release of monoamines by caffeine, if it occurs, could down-regulate monoamine receptors in the brain.

METHODS AND MATERIALS

Animals and administration of caffeine. For all experiments, we used female BALB/cBy mice, 4 months of age, weighing 19-21 g, from the breeding colony of our institute. The animals were kept on a 12-hour light/dark cycle (7 a.m./7 p.m. light); with food and water available ad libitum. Animals in chronic experiments were housed in groups of six. Caffeine was administered for three weeks via the drinking water; a control group of the same age received water without caffeine. The consumption of water and the body weights of the animals were measured every 3 or 4 days; fresh caffeine solutions were prepared twice a week. Three different dosage groups had access to water containing 0.3, 0.5, and 1.0 mg of caffeine per ml. Taken together with measures of body weight and water intake, this resulted in approximate doses of 73, 123, and 162 mg/kg per day. Twenty-four hours before the measurement of receptors, the animals were taken off the caffeine to allow a wash-out of the drug from the circulation.

Uptake of [^{3}H]dopamine and [^{3}H]serotonin into synaptosomes. Freshly dissected parts of the brain were processed for uptake measurements in the presence of various concentrations of caffeine as described previously (13,14). Uptake of [^{3}H]dopamine (23.1 Ci/mmol; New England Nuclear Corp., Boston, MA) and [^{3}H]serotonin binoxalate (26.7 Ci/mmol; New England Nuclear Corp.) was terminated after 2 min and 4 min, respectively, by filtration through Millipore filters (0.65 μm). The final concentrations of dopamine and serotonin in the uptake assays were made 0.1 μM by addition of unlabeled compound. Neuronal uptake of [^{3}H]dopamine by the dopamine carrier in the striatum was defined as the difference between the total uptake and the nonspecific uptake in the presence of 50 μM benztropine. As a measure for the norepinephrine carrier in the hypothalamus we used the difference between the total uptake of [^{3}H]dopamine and the nonspecific uptake with 1 μM desipramine (15), and for the serotonin carrier in the striatum the difference between total uptake of [^{3}H]serotonin and nonspecific uptake with 50 μM chlorimipramine. Filters and incubated samples were assayed for radioactivity as described previously (13).

Release of monoamines from synaptosomes. For the measurement of release from synaptosomes in S_1 fractions the method of Cardinali (16) was adopted. Tissue was homogenized in 0.25 M sucrose containing 0.1 mM EDTA; after centrifugation at 1,000 *g* for 10 min, the supernatant (S_1)

was obtained. The S_1 fraction was diluted with Krebs-Ringer buffer (for composition see ref. 14) containing various concentrations of caffeine, and uptake of monoamine was initiated by addition of [^{3}H]dopamine or [^{3}H]serotonin (0.1 μM final concentration). Ten min later, synaptosomes were depolarized by addition of 30 mM KCl (final concentration). Three min after the KCl addition, the incubated samples were filtered through Millipore filters (0.65 μm).

For the measurement of release from P_2 fractions, S_1 fractions prepared and diluted with Krebs-Ringer buffer as described above were preloaded with [^{3}H]dopamine or [^{3}H]serotonin (0.1 μM final concentration) at 37°C for 20 min. The S_1 fractions were centrifuged at 5,000 *g* for 10 min and the resulting P_2 pellet was resuspended in ice-cold Krebs-Ringer buffer. Within a few min, 0.2-ml aliquots of this suspension (equivalent to approximately 4 mg of fresh tissue) were pipetted into tubes containing 0.8 ml of Krebs-Ringer and various concentrations of caffeine, with or without KCl (30 mM final concentration), at 37°C. After 3 min, synaptosomes were collected by filtration through Millipore filters (0.65 μm); control samples were filtered at zero time.

Nonspecific uptake of [^{3}H]dopamine in the striatum and hypothalamus was defined with 50 μM benztropine and 1 μM desipramine, respectively, and was deducted.

Receptors for monoamines. Membranes from the cerebral cortex were assayed as described previously for α_1-adrenergic receptors (17) and 5-HT_2 receptors (18,19). Fresh cerebral cortex tissue pooled from two mice was homogenized with a Brinkmann Polytron (setting 6, 15 sec) in 40 vol of ice-cold 50 mM Tris-HCl buffer, pH 7.4 at room temperature, containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$. The homogenate was divided into two portions and centrifuged at 30,000 *g* for 10 min. The supernatant was discarded, one pellet was homogenized with the Polytron in 50 vol of the Tris buffer as described above, and the other pellet was homogenized in 50 vol of ice-cold 50 mM Tris-HCl buffer, pH 7.7 at room temperature. Aliquots of the former homogenate (approximately 1 mg of protein) were incubated in a final volume of 2 ml of Tris buffer with the ions as described above for 60 min at 20°C in the presence of 0.2 nM [^{3}H]spiroperidol (25.1 Ci/mmol; New England Nuclear). Nonspecific binding was defined with 0.1 μM mianserin for the estimation of 5-HT_2 receptors. Aliquots of the latter homogenate (approximately 1 mg of protein) were incubated in a final volume of 2 ml of 50 mM Tris-HCl, pH 7.7, for 30 min at 25°C in the presence of 0.5 nM [^{3}H]WB-4101 (17.6 Ci/mmol, New England Nuclear). Nonspecific binding was defined with 100 μM norepinephrine for the estimation of α_1-adrenergic receptors. Assays were terminated on GF/B glass fiber filters (17-19) with a Brandel cell harvester.

Membranes from the striatum were assayed for D_2 dopamine receptors as described previously (20). Fresh striatal tissue, pooled from two mice, was homogenized in 10 vol of ice-cold Tris buffer with ions as described above in a glass homogenizer. Aliquots of the homogenate (approximately 0.2 mg of protein) were incubated in a total volume of 2 ml of Tris buffer with ions as above for 2 h at 20°C in the presence of 0.1 nM [^{3}H]spiroperidol. Nonspecific binding was defined with 1 μM (+)-butaclamol. Assays were terminated as described above.

The protein content of the final membrane suspensions was estimated by the method of Lowry et al. (21). Differences between groups were tested for statistical significance with the two-tailed Student's t-test ($p=0.05$).

RESULTS

Uptake of monoamines into synaptosomes. [^{3}H]Dopamine was used to measure the activity of the dopamine carrier in the striatum and the norepinephrine carrier in the hypothalamus. These carriers were not inhibited by concentrations of caffeine ranging from 10^{-7} M to 10^{-3} M (Fig. 1). Very high concentrations of caffeine became inhibitory with IC_{50} values of approximately 10^{-2} M. The serotonin carrier, measured in the striatum with [^{3}H]serotonin, was inhibited by slightly lower concentrations of caffeine with an IC_{50} value of about 2×10^{-3} M. Concentrations of caffeine higher than 10^{-2} M inhibited not only the chlorimipramine-sensitive uptake of [^{3}H]serotonin, but also the chlorimipramine-insensitive uptake into compartments other than serotonergic nerve terminals (data not shown).

Release of monoamines from synaptosomes. A modest release of 21% in 3 min was observed by the addition of 30 mM KCl to striatal S_1 fractions preloaded with [^{3}H]dopamine (Fig. 2, left panel). Concentrations of caffeine ranging from 10^{-5} to 10^{-3} M, present from the start of the preloading, had no effect on the apparent release of [^{3}H]dopamine. Since uptake of [^{3}H]dopamine is not affected by these concentrations of caffeine (Fig. 1), it is legitimate to conclude that caffeine did not affect dopamine release under the conditions used. Only 10 mM caffeine delayed the apparent release somewhat (Fig. 2, left panel); the delay of true release is perhaps underestimated because at this concentration caffeine also inhibits uptake of [^{3}H]dopamine (Fig. 1).

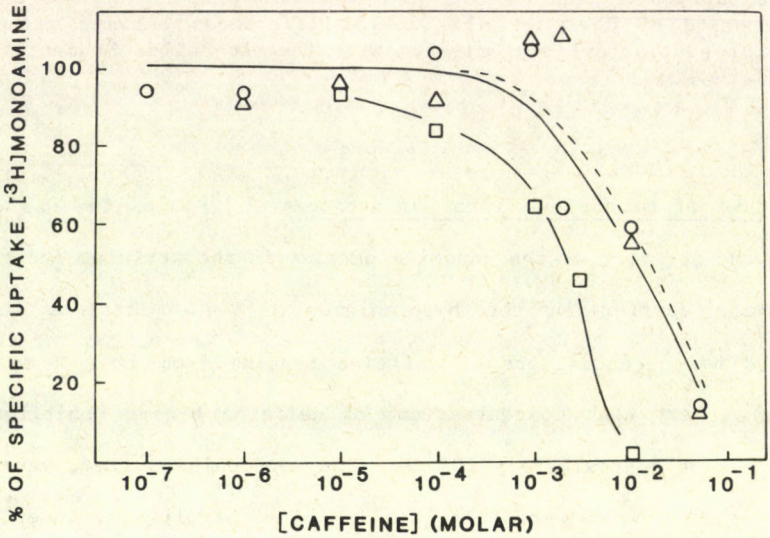

Fig. 1. Effect of caffeine on the uptake carrier for dopamine (DA), norepinephrine (NE), and serotonin (5-HT). Neuronal uptake was measured as described in "Methods and Materials". Each point is the average of triplicate measurements □——□, 5-HT striatum; △- - - -△, NE hypothalamus; and ○——○, DA striatum.

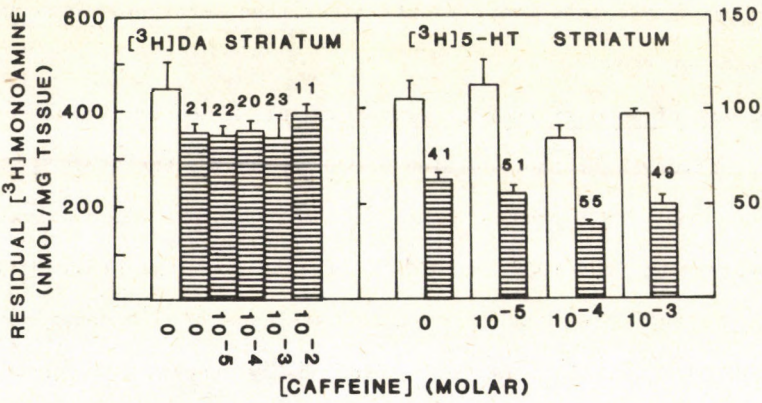

Fig. 2. Effect of caffeine on in vitro KCl-induced release of [^{3}H]dopamine (DA) and [^{3}H]serotonin (5-HT) from striatal synaptosomes in S_1 fractions. S_1 fractions were preloaded with [^{3}H]monoamine and release was initiated with 30 mM KCl. Release was terminated after 3 min. All results are averages of triplicate measurements ± standard error of the mean. Open bars denote incubations in the absence of KCl; hatched bars incubations in the presence of 30 mM KCl. The numbers above the hatched bars represent the release as percent of the total radioactivity remaining in the preparation after incubation in the absence of KCl. There were no statistically significant differences (Bonferroni-adjusted two-tailed Student's t-test) between the release values at 0, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M caffeine.

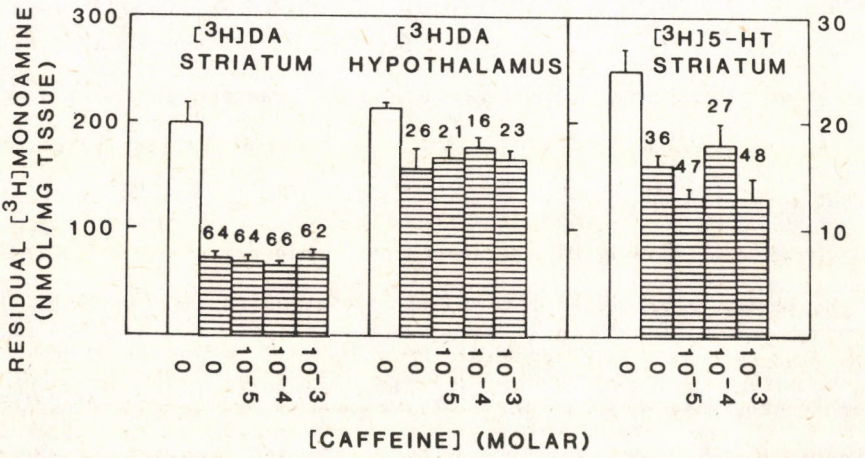

Fig. 3. Effect of caffeine on in vitro KCl-induced release of [^{3}H]dopamine (DA) and [^{3}H]serotonin (5-HT) from striatal and hypothalamic synaptosomes in P_2 fractions. After preloading, excess of [^{3}H]monoamine was removed by preparing P_2 fractions. Release was initiated by adding aliquots of the P_2 fraction to tubes containing 30 mM KCl. Incubation mixtures were filtered immediately (open bars) or 3 min later (hatched bars). Otherwise as Fig. 2. There were no statistically significant differences (Bonferroni-adjusted two-tailed Student's *t*-test) between the release values at 0, 10^{-5}, 10^{-4}, and 10^{-3} M caffeine.

Since the uptake of [^{3}H]serotonin into striatal synaptosomes is affected by concentrations of caffeine above 10^{-4} M (Fig. 1), we compared the release of [^{3}H]serotonin induced by KCl with that observed in the absence of KCl at each concentration of caffeine (Fig. 2, right panel). Indeed, at 10^{-4} and 10^{-3} M caffeine the S_1 fractions retained lower amounts of [^{3}H]dopamine during the preloading and release phase than those retained by S_1 fractions in the absence of caffeine (open bars). The amount of [^{3}H]serotonin released by KCl in 3 min was 41% of the total amount of [^{3}H]serotonin retained by the S_1 fraction in the absence of KCl; in the presence of 10^{-5} to 10^{-3} M of caffeine release values of 49 to 55% were observed.

In another series of experiments the [^{3}H]dopamine and [^{3}H]serotonin in the medium was removed before the initiation of release by KCl (Fig. 3). The release of [^{3}H]dopamine from striatal synaptosomes (64%) and from hypothalamic synaptosomes (26%) was unaffected by concentrations of caffeine between 10^{-5} and 10^{-3} M (Fig. 3, left panel). In addition, little or no effect of caffeine was observed on the release of [^{3}H]serotonin from striatal synaptosomes (Fig. 3, right panel).

Receptors for monoamines. There were no differences in body weights between animals treated with 73 mg/kg per day of caffeine in their drinking water and control animals on normal water at any point during the experiment (Table I). The water consumption was initially the same for the two groups of animals; during the second and third week the caffeine group drank more water than the control group. The amount of caffeine consumed was approximately 66 mg/kg per day in the first week, and 76 mg/kg per day in the second and third week. This dose of caffeine did not result in significant change in D_2-dopaminergic, 5-HT_2-serotonergic, or α_1-noradrenergic receptors in the striatum and cerebral cortex (Table I).

TABLE I

EFFECT OF CHRONIC CAFFEINE ADMINISTRATION ON MONOAMINE RECEPTORS IN MOUSE BRAIN[a]

Measure	Control	Caffeine 73 mg/kg day	Change
Body weight on day 1 (mg)	19 ± 0.4[b]	19 ± 0.3	0%
Body weight on day 8 (mg)	18 ± 0.4	19 ± 0.3	+ 5%
Body weight on day 15 (mg)	19 ± 0.4	19 ± 0.4	0%
Water consumption day 1-8 (ml/mouse/day)	4.0 ± 0.1[c]	4.2 ± 0.2	+ 5%
Water consumption day 8-15 (ml/mouse/day)	3.6 ± 0.3	4.8 ± 0.3	+33%[h]
Water consumption day 15-21 (ml/mouse/day)	3.8 ± 0.2	4.8 ± 0.4	+26%[h]
D_2 receptors[e] (fmol/mg prot)	176 ± 10[d]	156 ± 4	-12%
5-HT_2 receptors[f] (fmol/mg prot)	26.5 ± 1.6[d]	24.6 ± 1.8	-7%
α_1-adrenergic receptors[g] (fmol/mg prot)	45.5 ± 1.2[d]	42.2 ± 1.8	-7%

[a]The caffeine group received caffeine in the drinking water for 3 weeks; the control group had drinking water without caffeine. Caffeine was discontinued 24 h before receptor measurements. Each group consisted of 12 animals.

[b]All results, except where indicated, are means ± SEM.

[c]Water consumption was measured for combined groups of six animals; values shown are means ± range for two groups of 6 animals in each treatment group.

[d]Brain samples of two animals were pooled for each determination; values shown are means ± SEM for 6 pools. There were no statistically significant differences between the control group and caffeine group.

[e]The concentration of [^{3}H]spiroperidol was 0.12 nM.

[f]The concentration of [^{3}H]spiroperidol was 0.20 nM.

[g]The concentration of [^{3}H]WB-4101 was 0.43 nM.

[h]$p < 0.05$ two-tailed Student's *t*-test.

TABLE II

EFFECT OF CHRONIC CAFFEINE ADMINISTRATION ON MONOAMINE RECEPTORS IN MOUSE BRAIN[a]

Measure	Control	Caffeine 123 mg/kg/day	Caffeine 162 mg/kg/day
Body weight on day 1(mg)	19 ± 0.4[b]	19 ± 0.6 (0%)	21 ± 0.5 (+8%)
Body weight on day 7(mg)	19 ± 0.4	17 ± 0.5 (-11%)	18 ± 0.6 (-7%)
Body weight on day 14(mg)	19 ± 0.4	19 ± 0.6 (-1%)	18 ± 0.6 (-6%)
Body weight on day 21(mg)	20 ± 0.4	19 ± 0.7 (-4%)	18 ± 0.6 (-11%)
Water consumption day 1-7 (ml/mouse/day)	4.6 ± 0.2[c]	4.0 ± 0.2 (-13%)	3.1 ± 0.5 (-33%)[h]
Water consumption day 7-14 (ml/mouse/day)	4.6 ± 0.1	4.8 ± 0.0 (+3%)	3.1 ± 0.7 (-34%)[i]
Water consumption day 14-21 (ml/mouse/day)	4.5 ± 0.0	4.6 ± 0.1 (+1%)	2.8 ± 0.4 (-37%)[h]
D_2 receptors[e] (fmol/mg prot)	186 ± 8[d]	168 ± 8 (-9%)	185 ± 9 (0%)
5-HT_2 receptors[f] (fmol/mg prot)	35.5 ± 1.9[d]	38.7 ± 2.3 (+9%)	35.9 ± 2.4 (+1%)
α_1-adrenergic receptors[g] (fmol/mg prot)	52.5 ± 1.6[d]	55.1 ± 1.5 (+5%)	51.8 ± 2.4 (-2%)

[a] As in Table I. Percent change compared with control group is indicated between parentheses.

[b] All results, except where indicated, are means ± SEM.

[c] Water consumption was measured for combined groups of six animals; values shown are means ± range for two groups of 6 animals in each treatment group.

[d] Brain samples of two animals were pooled for each determination; values shown are means ± SEM for 6 pools. There were no statistically significant differences between the control group and the caffeine groups.

[e] The concentration of [^{3}H]spiroperidol was 0.14 nM.

[f] The concentration of [^{3}H]spiroperidol was 0.31 nM.

[g] The concentration of [^{3}H]WB-4101 was 0.59 nM.

[h] $p < 0.02$ two-tailed Student's *t*-test, with Bonferroni adjustment.

[i] $p < 0.05$ test as above.

Table II shows the results obtained with doses of caffeine of 123 mg/kg per day and 162 mg/kg per day. The control animals and those treated with 123 mg/kg per day of caffeine had a constant body weight throughout the 3-week period. In contrast, the higher caffeine dose of 162 mg/kg per day caused a drop in body weight in the first week. The water consumption of the control animals was constant over the 3-week period, whereas the group on 123 mg/kg per day of caffeine had a higher intake during the 2- and 3-week than during the 1-week period, reminiscent of the effect of the dose of 73 mg/kg per day (Table I). In contrast, the dose of 162 mg/kg per day resulted in a lower intake of water as compared to control animals at all times (Table II). There were no statistically significant differences between the three groups in D_2-dopaminergic, 5-HT-serotonergic, or α_1-noradrenergic receptors in the striatum and cerebral cortex (Table II).

DISCUSSION

No direct effects of caffeine were noted on uptake of monoamines into synaptosomes at physiologically relevant concentrations (Fig. 1). It appears that caffeine is less potent than theophylline or pentoxifylline in inhibiting synaptosomal uptake of monoamines (16). In the study by Cardinali (16) concentrations of 0.1 mM of theophylline and pentoxifylline appreciably inhibited monoamine uptake. The present results also show that caffeine is less potent than theophylline and pentoxifylline in modulating K^+-evoked release of monoamine neurotransmitters from synaptosomal preparations (16). Concentrations of caffeine up to 1 mM had no effect on *in vitro* K^+-induced release of [^{3}H]serotonin from serotonergic nerve terminals in the striatum, of [^{3}H]dopamine from dopaminergic nerve terminals in the striatum, and of [^{3}H]dopamine from noradrenergic

terminals in the hypothalamus (Figs. 2 and 3). The present experiments did not address the possibility of the need for the presence of adenosine agonists, which by occupying adenosine receptors can inhibit monoamine release (22); this might uncover an effect of caffeine in disinhibiting release by blocking adenosine receptors. This approach has proven difficult in studies on acetylcholine release from brain synaptosomes: an endogenous factor(s) (substance B, see ref. 23) appears to mask presynaptic purinergic receptors on cholinergic nerve terminals. The present results clearly show the inability of caffeine to directly modulate monoamine release at concentrations orders of magnitude higher than those required in brain to induce behavioral effects in rats (24). We should not dismiss the possibility that releasing effects of methylxanthines other than caffeine play a role in their central stimulatory action. For instance, 8-phenyl-theophylline is capable of increasing the release of amino acid neurotransmitters from brain slices evoked by electrical stimulation at 0.2 Hz (8). In addition, theophylline, 8-phenyl-theophylline, and 3-isobutyl-1-methyl-xanthine stimulate, be it only modestly, norepinephrine release evoked by 3 Hz electrical stimulation (6).

Changes in uptake or release of monoamines by caffeine could possibly alter the amount of neurotransmitter present in the synaptic cleft. Such presynaptic effects, if present in vivo, could result in long-term changes in monoamine transmitters acting on postsynaptic monoamine receptors during chronic administration of caffeine. Experiments were therefore carried out to assess possible changes in monoamine receptors in animals treated for 3 weeks with caffeine in their drinking water at a low dose (73 mg/kg per day), an intermediate dose (123 mg/kg per day), and a high dose (162 mg/kg per day). The higher water intake observed with the low and intermediate doses of caffeine (Tables I and

II) could reflect caffeine's antidiuretic effect. The high caffeine dose was probably close to producing toxicity in our animals, since it caused a reduction in water intake at all times during the experiment and a reduction in body weight in the first week (Table II). An alternative possibility is that the high caffeine concentration in the drinking water (ca 1 mg/ml) was perceived as aversive by the mice in the high dose group. There were no changes in α_1-adrenergic receptors in the cerebral cortex, 5-HT_2 receptors in the central cortex, or D_2 receptors in the striatum in any caffeine group compared to control animals (Tables I and II). This suggests that there were no persistent changes in monoamine neurotransmitter concentrations acting upon monoamine receptors, consonant with the lack of presynaptic effects observed in the series of experiments presented above. Higher doses of caffeine than those administered in the present study are not likely to give relevant information, and results would be difficult to interpret because of toxicity problems. Chronic administration of caffeine has been shown to be ineffective in regulating benzodiazepine receptors (25) or adenosine uptake sites (25). So far, the only receptor system affected seems to be the adenosine receptor, which shows up-regulation upon chronic treatment with caffeine (25-27). The lack of effect of chronic caffeine administration via drinking water on monoamine receptors in the present study is consonant with the finding that chronic caffeine administration from subcutaneous reservoirs did not alter dopamine or norepinephrine level and turnover rate in various brain regions; also unaffected were levels of metabolites of dopamine, norepinephrine, and serotonin (28). All these results taken together suggest that caffeine has only a mildly modulatory effect in brain, and that caffeine should not be grouped within one category with other, more potent psychostimulant drugs with different

mechanisms of action. Results from behavioral experiments also underscore differences rather than similarities between caffeine and other psychostimulants. For instance, infusion of muscimol into the region of nucleus accumbens efferent terminals blocks amphetamine- but not caffeine-induced locomotion (29). A high dose of naloxone can indirectly antagonize amphetamine-induced locomotion, but has no effect on caffeine-induced hyperactivity (30). Furthermore, caffeine maintains its locomotor-activating property following destruction of dopaminergic terminals within the nucleus accumbens by 6-hydroxydopamine (31). There is less similarity in the activating property between caffeine and amphetamine than between caffeine and corticotropin-releasing factor (CRF) (29). It is possible that caffeine-induced activation have some common elements with stress-induced behavioral activation involving release of CRF. The neurochemical results presented in this paper are consonant with the differences between behavior caused by caffeine and that caused by other psychostimulants in that caffeine does not have the effects on monoamines displayed by amphetamine and cocaine, such as the inhibition of monoamine uptake, the stimulation of monoamine release, and the regulation of monoamine receptors.

ACKNOWLEDGEMENTS

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FROM METHYLGLYOXAL TO NEW IMMUNOPOTENTIATING ASCORBIC ACID DERIVATIVES[*]

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In Szent-Györgyi's search for α-dicarbonyl compounds that play an important role in cell regulation, 3-desoxyglucosulose was isolated first from liver but did not prove active. Methylglyoxal came next, however, its toxicity prompted Szent-Györgyi to suggest a combination with ascorbic acid which, indeed led to immunopotentiating enediol acetals although of low stability. Therefore the vinylogue of methylglyoxal, acetylacrolein was coupled with L-ascorbic acid carbanion. This second new reaction, of the aldol-type, led to the stable, potent immunoactive compound, 2-(5-methylfuryl)-3-ketogulonolactone cyclohemiketal that forms a completely surprising H-bond with succinic anhydride and succinimide based on an X-ray study. A third new reaction in which ascorbic acid plays the role of a Michael donor to α,β-unsaturated aldehydes and ketones proved now to be of general validity; it is unexpectedly acid catalyzed and the adducts formed with aliphatic and alicyclic olefin ketones have definite immunopotentiating effect. A brief description of the biological effects of all types of new compounds is outlined.

*Senior author to whom correspondence is to be addressed. Ph.D. from Szeged University, (Organic Chemistry, 1937) under A. Szent-Györgyi. He dedicates this paper to the memory of the man whom he owes his scientific perspective and with whom he cooperated until his death.

Abbreviations: AA, L-ascorbic acid; MG, methylglyoxal.

[*] *In memoriam Albert Szent-Györgyi*

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INTRODUCTION

Although this paper contains mostly organic chemical material and as such would not fit into the general pattern of this ACTA, the 50 years long experience of one of us (G.F.) with Albert Szent-Györgyi is a testimony of his appreciation and support of organic chemistry. To restrict references to cooperative efforts in recent years, Szent-Györgyi in 1967, was searching for a dicarbonyl compound that plays a major role in cellular regulations. The first compound he isolated from liver tissue, proved to be an α-ketoaldehyde, namely 3-deoxyglucosulose (Fodor, Sachetto, Szent-Györgyi and Együd, 1967) that, although biologically inactive, was found to be a natural product (Kato et al., 1970) at variance with the assumption (Osuka and Együd, 1968) that it is an artifact.

METHODS

The reaction of methylglyoxal with L-ascorbic acid (AA) was carried out either in water or in anhydrous tetrahydrofuran, at 20°. The reaction was monitored first by the decreasing iodine consumption, i.e. disappearance of the enediol group of AA. Later HPLC was applied, using the Varian Model LC 5000 with a reverse-phase MCH-10 column as an analytical tool and the Waters Model Auto 500 on a preparative scale. HPLC gave direct information on both consumption of AA and formation of product(s) of Type 1. Isolation of the new AA derivatives was achieved by freeze-drying on a Virtis Freezemobile 24. Characterization of the product was done by ^{1}H NMR spectrometer, Varian EM-360 at 60 MHz; ^{13}C NMR spectra were taken on a Varian CFT-20 spectrometer at 20 MHz. IR spectra were recorded on the Beckman IR 12 spectrophotometer. Optical rotations were measured on a Perkin-Elmer Model 141 electric polarimeter, mass spectra on a Finnigan 4021 mass spectrometer with an INCOS data system. Elemental analyses were made by Galbraith Laboratories, Inc., Knoxville, TN. These physical methods were universally applied to all three new reactions of AA.

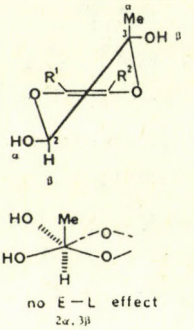
The aldol reaction was carried out by adding the cyclic acetal of the unsaturated 1,4-dicarbonyl compound, i.e., 2-methyl-2,5-dimethoxy-2,5-dihydrofuran to a concentrated aqueous solution of AA without prior hydrolysis of the acetal to the dioxo compound, at 20°; the furyl ketogulonolactones form in a few hours; isolation followed by freeze drying, recrystallization or in the case of 4 by crystalline complex formation with succinic anhydride.

The Michael addition of AA to α,β-unsaturated ketones was aided by a catalytic addition of protic acid in water, that gave mostly crystalline adducts which separated, e.g., compounds 10, 13, 14, or 9 that was obtained upon freeze-drying of the aqueous solution. Compounds 5, 7, 8,

10, and the cyclic trimer of 9 (Arnold, Fodor, George and Karle, 1987) were subjected to X-ray structure determination at the Laboratory for the Structure of Matter, Washington, DC, by Drs. Isabella Karle, Judith Flippen-Anderson and Clifford George, respectively.

RESULTS AND DISCUSSION

After 3-desoxyglucosulose, Szent-Györgyi's attention was focused on methylglyoxal (Szent-Györgyi, 1978). Again, he isolated methylglyoxal from beef liver tissue which was identified by comparing its 2,4-dinitrophenylhydrazone derivative with that of a pure synthetic product (Fodor, Mujumdar, Szent-Györgyi, 1978). However, the relatively high toxicity of methylglyoxal prevented therapeutic experiments so Szent-Györgyi suggested to one of us (GF) to try to couple it with L-ascorbic acid. Unexpectedly, the enediol group reacted with the ketoaldehyde to form an amorphous mixture of regio and stereoisomers of MG-AA while the reductive power of ascorbic acid against iodine disappeared (Szent-Györgyi and Fodor, 1978, 1979). A conformational analysis of the products showed that only two (1,2) out of the eight possible isomers would not exert a Edward-Lemieux effect (Fodor, 1982; Arnold, Fodor, Mathelier, Mohácsi, Szent-Györgyi, Veltri, 1983); interestingly an immunopotentiating effect was detected (Veltri, Fodor, Mathelier, 1982). Also, the MG-AA mixture was 93-96% active against Ehrlich Ascites carcinoma (Elvin, Slater, 1981). Unfortunately, MG-AA proved unstable in aqueous solutions; it underwent hydrolysis. According to Szent-Györgyi (1978) the electron-withdrawing property of the methylglyoxal carbonyls seemed essential. However, we decided to introduce a vinyl group between the two carbonyl functionalities which should maintain electron attraction; even more so, since it forms a channel for the flow of electrons between the two carbonyls thus the unsaturated 1,4-dicarbonyl compounds form much more stable products with ascorbic acid. It turned out that, indeed, by using maleic aldehyde or its homolog cis-β-acetylacrolein (3) stable products were obtained (Szent-Györgyi and Fodor, 1983, 1986) that have a much stronger immunopotentiating effect (Veltri, Fodor, Liu, Baseler, 1984) than the derivative of methylglyoxal. Unlike methylglyoxal, however, ascorbic acid did not react with the enediol hydroxyl(s) but acted as the 2-carbanion donor, in an aldol-type reaction with the unsaturated dicarbonyl compounds. The structure of the product with cis-β-acetyl-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R2
HO
H
HO
Me
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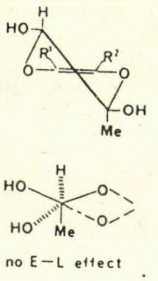
H
no E—L effect
2α, 3β

1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HO
R1
R2
OH
Me
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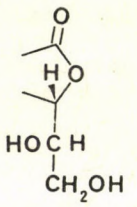
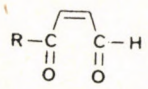
HO
HO
Me
no E—L effect

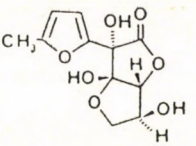
2

$R^1 + R^2 =$ 
O
H O
HO C H
CH2OH



R = H
= CH_3

3



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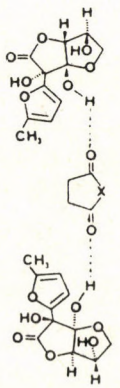
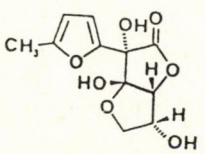


5 X : O
X : NH
X : NCH_3



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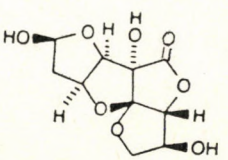

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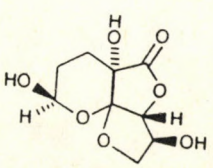

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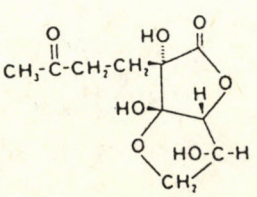

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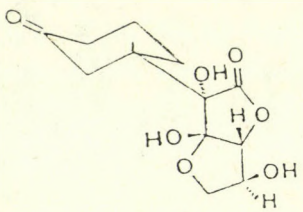

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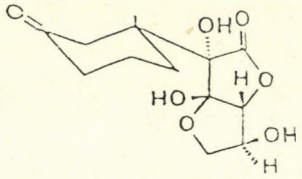

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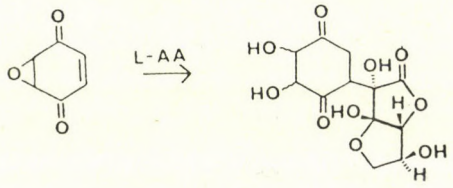

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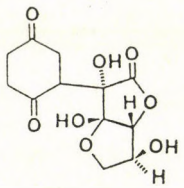

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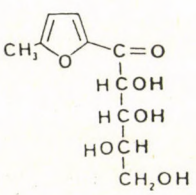

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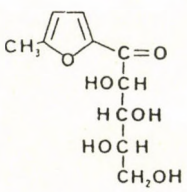


16

acrolein or with its cyclic acetal-ketal, namely 2-methyl-2,5-dimethoxy-2,5-dihydrofuran was crystalline and formed through a stereospecific process: it proved to be 2-(5-methylfuryl)-3-ketogulonolactone <3,6>hemiketal (4). This product gave a completely unknown type of H-bonded complex with the carbonyl groups of succinic anhydride and succinimide (5), respectively (Fodor, Sussangkarn, Arnold, Karle and George, 1983; Fodor, Sussangkarn, Mathelier, Arnold, Karle, and George, 1984). It was found that this complex formation is highly substrate specific. No other AA derivative gave an adduct and also 2,4-thiazolidinedione can be added as acceptor to the succinic derivatives listed under formula 5 (Sussangkarn, George and Karle, 1986, unpublished).

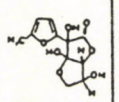
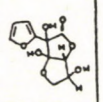
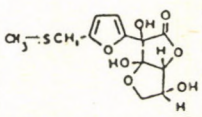
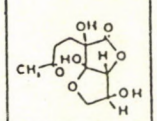
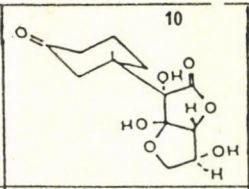
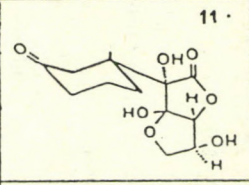
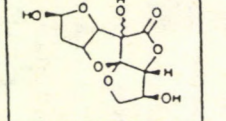
Maleic aldehyde gave with ascorbic acid the analogous nor-compound (Chart I). This new reaction of L-ascorbic acid proved of general validity: all furan derivatives that are convertible into 2,5-dimethoxy-2,5-dihydrofurans react with L-ascorbic acid in a similar fashion. Also the methylthiomethylfuryl derivative has been synthesized and its biological activities tested (17, Chart I).

According to Sussangkarn (1986) D-isoascorbic acid, the C-5 epimer of L-ascorbic acid reacts with cis-β-acetylacrolein in an analogous fashion (6) but no H-bonded complex was isolated. However, by changing the stereochemistry of the cis-1,4-dicarbonyl olefin to trans results in an entirely different reaction mechanism and gives a saturated tetracyclic butyrolactone derivative (7) (Fodor, Sussangkarn, Mathelier, Fang and Arnold, 1986).

Szent-Györgyi has observed in the early days of these investigations the formation of a crystalline precipitate from ascorbic acid and acrolein. Three of us have followed this lead and have isolated first an adduct and upon dehydration of the latter a new gulonolactone derivative that resulted from a Michael addition of the 2-ascorbate carbanion onto the α,β-unsaturated aldehyde. X-ray crystallography proved that the Michael adduct, through a concerted double cyclization gave rise to a tricyclic, saturated lactone (8) (Fodor, Arnold, Mohácsi, Karle and Flippen-Anderson, 1983). That compound has only a weak immunomodulatory effect (Veltri, unpublished, 1986). However, methyl vinyl ketone, acrolein's methyl homolog gives a ketobutyl-gulonolactone cycloketal (9)

CHART I

STRUCTURE/ACTIVITY RELATIONSHIPS FOR ASCORBIC ACID DERIVATIVES

	4  MFBL	nor 4  FBL	17.  TMFBL	9  KBBL	10  KCBL-A	11 ·  KCBL-B	7.  AAFA
LSA-CON-A	++	++	+	+++	+	+++	++
LSA-PHA	++++	++++	+++	++	++	++	++
JERNE-TD	+++	++++	+++	++	++	++++	+++
JERNE-TI	(-)	(-)	(-)	(-)	NT	NT	NT
IL-2	+++	(-)	(-)	++++	+++	++	NT
INTERFERON	++++	(-)	(-)	++	+	++++	NT
ANTITUMOR ACTIVITY	++	NT	NT	++	NT	NT	NT
NK (YAC-1)	+	NT	NT	+	NT	NT	NT
MACs-SOA	+	(-)	(-)	(-)	(-)	(-)	NT
MACs-IL-1	+	NT	NT	NT	NT	NT	NT
PMNs	++	NT	NT	NT	NT	NT	NT

\+ to ++++ = Degree of amplification
(-) = No amplification
NT = Not tested

that is immunoactive (Veltri, Baseler, Fodor, Sussangkarn, Maxim, 1986; Baseler, Veltri, Fodor, Sussangkarn, Maxim, 1987).

Quite recently we found that much less reactive Michael acceptors than methyl vinyl ketone can be brought into reaction with ascorbic acid if acid catalysis is applied. For example 2-cyclohexenone and 2-cyclopentenone gave well-defined crystalline products (Sussangkarn and Fodor, 1986). With 2-cyclohexenone two diastereoisomeric products formed (10,11), different in the configuration of C-3' of the alicyclic moiety. The absolute configurations of these stereoisomers have been assigned, first by applying the octant rule (Moffitt, Woodward, Moscowitz, Klyne, Djerassi, 1961) by using a self-constructed octant cage into which Dreyding models of the stereoisomers could be fitted in. The first crop showed in the partial ORD curve a negative Cotton effect while the second modification indicated a positive Cotton effect (Sussangkarn and Fodor, 1986). It was concluded that the first crop was the 2-(1'-keto-3'S-cyclohexyl)-3-keto-L-gulonolactone, while the second, but more immunoactive modification has the 3'R configuration. These assignments have been corroborated by X-ray crystallographic data (Sussangkarn, Fodor, George and Karle, 1987).

A new idea is to construct an immunopotentiating molecule that at the same time may contain an antibacterial or antiviral prosthetic group such as a quinonoid structure. Since the parent p-benzoquinone and ascorbic acid undergo oxidation-reduction reaction due to the high redox potentials, quinone monoepoxide (12) was synthesized (Alder, Flock and Beumling, 1960) and was allowed to react with ascorbic acid. The product proved to be the Michael adduct of ascorbic acid to the olefinic double bond of benzoquinone 2,3-epoxide on C-5,6, followed by hydrolytic ring opening of the epoxide function to give the corresponding 2-(1',4'-diketo-2',3'-trans-dihydroxy-5'-cyclohexyl)-3-keto-gulonolactone<3,6>cyclo-hemiketal (13) (Sussangkarn and Fodor, 1987).

Another quinone-related model reaction partner to AA was 2,3-dihydrobenzoquinone-1,4, that is 2-cyclohexene-1,4-dione, which reacted smoothly with ascorbate carbanion under acid catalyzed conditions via the Michael reaction to (14) (Sussangkarn, 1986). D-isoascorbic acid did not show any striking difference in reactivity towards α,β-unsaturated ketones, although the immunological screening of these last mentioned

compounds still awaits its turn.

The last segment shall give an overview of the biological activity of the compounds arising from both the aldol and the Michael type reactions of L-ascorbic acid. The former field was recently covered (Veltri, Fodor, Liu, Woolverton and Baseler, 1986). Concerning the studies on the metabolism of methylfuryl-ketogulonolactone (4) we have a few interesting preliminary observations before a more systematic tracer study can be carried out. One of us (RV) succeeded in isolating small amounts of an amorphous compound from the urine of experimental animals that have been treated with the furyl-gulonolactone derivative. Upon our request Dr. Csaba Horváth and van der Schrieck (1984) at Yale University had carried out the preparative HPLC separation thereof into two components that were considered first as decarboxylation products that have recyclized to a pyranoside sugar. However, our CMR study (1985) showed that the two products of decarboxylation are probably epimeric furyl-ketohexoses, e.g. (15) and (16) (Sussangkarn and Fodor, 1985).

IMMUNOLOGICAL SURVEY

As indicated before, the new chemical adducts and condensates of Vitamin C have resulted in at least two new classes of potentially useful synthetic immunopharmaceuticals. The first class of reaction is referred to as the methylfurylbutyrolactones (code MFBLs), chemically known as 2-(5-methyl-2-furyl) 3-keto-4-(5,6-dihydroxyethyl) butyrolactone<3,6> hemiketal, synonym for gulonolactone 4, and its molecular complexes (5) produced with succinic anhydride and succinimide (Fodor, Sussangkarn, et al., 1984). The immune amplifying capabilities of these compounds are described, in part, by Veltri, Fodor, Liu, et al. (1986). Employing classical methods of cellular immunology in murine models, we demonstrated that the MFBLs when given to mice via intraperitoneal, intravenous or oral routes primes T-lymphocytes to produce a heightened response to either non-specific polyclonal mitogens (i.e., plant lectins) or specific T-cell dependent antigens. Such MFBL treated and activated lymphocytes undergo a significantly elevated proliferative response, with DNA synthesis being increased 5-7 times over controls. Similarly, such MFBL primed lymphocytes when triggered with concanavalin-A in vitro yield several fold

more lymphokines including interferons and interleukin 2 (Veltri, Baseler, Fodor, et al., 1987). A similar amplification of the antibody response to T-cell dependent antigens has been demonstrated by Maxim, Veltri, Baseler, et al. (1986). Employing either sheep erythrocytes or keyhole limpet hemocyanin as T-dependent antigens, and MFBL treatment of mice, at least 3-5 times more antibody producing lymphocytes were detected by the Jerne hemolytic direct plaque assay for complement dependent IgM producing B-lymphocytes. Dr. Maxim showed that the MFBLs were not non-specific polyclonal activators, but rather they were affecting the antigen specific immune response. Later he showed that MFBL does not amplify the antibody response to true T-cell independent antigens such as LPS-TNP (unpublished observations). This ability for MFBL to promote antibody production could prove very useful to monoclonal antibody technology and also be of value for improving the efficiency of new human or veterinary vaccines.

In regard to experimental anticancer research, early work was performed under a collaborative effort between Veltri and Wheelock (1984) using the L5178Y lymphoma "tumor dormant state" (TDS) model. The MFBLs were able to cure the ascites TDS as evidenced by tumor cell counts and survival statistics (Wheelock, Veltri, Liu, et al., 1984). Subsequently, Veltri, Maxim, Baseler, et al. (1984) demonstrated that 200 mg/kg of MFBL when combined with a single dose of cyclophosphamide (20 mg/kg) significantly ($p < 0.048$) extended the life span (+78%) of mice bearing the Lewis Lung carcinoma. Further investigations at the American Biotechnology Company demonstrated that 100 mg/kg of MFBL given for seven days significantly ($p < 0.01$) inhibits lung metastases of the B-16(F10) line of melanoma in C57Bl/6 mice (submitted to the J. Natl. Cancer Institute, 1987). Finally, Maxim, Baseler, Cameransi, et al. (1987) reported that 200 mg/kg 5 and 5-fluorouracil (5-FU) given for nine days to mice bearing the L1210 leukemia survived 50% longer than those treated only with 5-FU ($p < 0.039$). The research on cancer to date supports the probable utility of this class of immunomodulators for adjunctive treatment of human cancer at some future date.

Another unique feature of the MFBLs was exhibited when human polymorphonuclear (PMN) cells were exposed to µg quantities in vitro. Woolverton, Veltri and Snyder (1986) demonstrated enhanced adherence, chemotaxis, phagocytosis, and intracellular microbial killing by PMNs

exposed to MFBL in vitro. More recently, Woolverton, Veltri and Snyder (1987) extended the observation to a mouse model and MFBL at 50 mg/kg produced recruitment of PMNs which manifested an increased resistance of the MFBL treated mice to infection with Streptococcus pneumoniae and Salmonella typhymurium. Such an anti-infective property of the MFBLs could eventually be of possible value for the treatment of severe acute infections of unknown etiology, chronic infections complicating malignancy or metabolic and genetic disorders, as well as management of infection in trauma or severe burn patients.

In 1986, under funding provided by a Small Business Innovative Research contract from the National Cancer Institute awarded to Veltri and Fodor, a second class of condensates of ascorbic acid, the ketone ascorbic acid condensates (KAACs) were discovered to be active. These reaction products were produced as a result of C-2 position of ascorbic acid serving as a Michael carbanion donor to α,β-unsaturated aldehydes and ketones (Fodor, Arnold, Mohacsi, et al., 1983). Baseler, Veltri, Fodor, Sussangkarn, et al. (1987) reported at the 71st annual Federation of American Societies for Experimental Biology that the KAACs amplified T-lymphocyte proliferation, lymphokine production, antibody production and often at lower doses. Also, one of the derivatives, KBBL (9), has demonstrated the ability to inhibit lung metastases in the B-16(F10) line melanoma model at doses of 100 mg/kg ($p < 0.01$) and 200 mg/kg ($p < 0.05$). The range of biological response modifications produced by the KAACs and their possible utility in the treatment of human disease is yet to be determined.

To provide an overview of the structure-function relationships observed to date on the MFBLs and KAACs, Chart I lists biological response modifications assayed in the very first column. The chemical structures are shown across the top of the chart. Structures 4, nor-4, and 17 are MFBL derivatives while 9, 10, 11 and 7 are considered KAACs. The chart only provides a qualitative perspective and there remain many areas of research to be completed. It remains the mission of the American Biotechnology Company to use the potential of these new synthetic pharmaceuticals for treatment of disease in man.

To summarize, it was a long way from Albert Szent-Györgyi's initial suggestion to start working with methylglyoxal, and then add ascorbic

acid, to the achievement of the synthesis of new type immunomodifying agents.

Also, the discovery of this new and fertile area of pharmaceutical chemistry is a tribute to the living memory of the true genius of Professor Albert Szent-Györgyi.

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THE NUMBER OF BINDING SITES : THE KLOTZ GRAPH ANALYSED*

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A semilogarithmic plot (bound versus log of free ligand) has been proposed (Klotz, Science 217, 1247, 1982) to replace advantageously the Scatchard plot. We show that this representation has its own serious practical limitations and conclude that there are no "perfect" solutions : data have to be analysed in the light of all possible models using all available representations.

Analysis of binding of a ligand to its binding site was reserved for a long time to the Happy Few. It was probably the discovery of steroid hormone receptors and the rapid expansion of radio-immuno-assays which caused the dramatic change in this situation : an exponentially growing number of scientists became suddenly interested to find a convenient formula for calculating binding constants and the number of binding sites. Most of them put their trust in the Scatchard plot (1) (The ratio of bound/free ligand plotted versus bound ligand), many appealed to more sophisticated models, nobody has found an universal solution, and leading authorities in the field continue debating this issue.

Titles and subtitles of recent papers are meaningful in this respect : Klotz (2) writes about "Facts and Fantasies" concerning the use and misuse of the Scatchard plot and suggests another : bound ligand plotted

*In memoriam Albert Szent-Györgyi

Akadémiai Kiadó, Budapest

against the logarithm of free ligand ; Munson and Rodbard (3) found necessary to present "A Constructive Critique" of Scatchard and Klotz graphs and praise the advantages of computerised approach based upon statistical analysis (see also the answer of Klotz (4)) ; Feldman presents the study of "Statistical Limits in Scatchard Analysis" (5), and - debating this latter approach - Klotz and Hunson (6) point to the existence of "Real Sites (and) Ghost sites"...

The Scatchard plot - because of its apparent simplicity - is now certainly the most popular method to calculate association constants and concentration of binding sites. No wonder that it has been widely misused, therefore warnings against misunderstandings are most welcome. Criticizing the general application of the Scatchard plot, Klotz (2) suggested a semi-logarithmic graph plotting bound ligand versus the logarithm of free ligand. Such a representation yields a sigmoidal curve. The curve representing the experimental data should pass through the inflection point, otherwise no conclusions about the concentration of binding sites can be reached.

We think that the exclusive use of the Klotz plot is not the ultimate solution and we want to show the vulnerable points of this approach. Further - in agreement with Munson and Rodbard (3) - we also think that experimental results have to be examined in the light of several possible models.

RESULTS AND DISCUSSION

Simple demonstration of the equations of a semi-logarithmic graph.

In the case of a ligand x binding to independant and equivalent sites one can write that, y, the saturation fraction ($0 \leqslant y \leqslant 1$)

is $$y = \frac{x}{1+x} \qquad \text{(Eq. 1)}$$

($x = K(X)$; K the affinity constant and (X) the concentration of free ligand)

This equation can be transformed in

$$y = \frac{e^s}{1 + e^s} \qquad \text{if } s = \ln x \ (\text{i.e } x = e^s) \qquad \text{(Eq. 2)}$$

One sees also that

$$\frac{dy}{ds} = y' = \frac{e^s}{(1+e^s)^2} = \frac{e^s}{(1+e^s)} \left[1 - \frac{e^s}{1+e^s}\right] = y\,(1-y) \qquad \text{(Eq. 3)}$$

and that

$$\frac{d^2y}{ds^2} = y'' = \frac{e^s}{(1+e^s)^2} \left[1 - \frac{2e^s}{1+e^s}\right] = y'\,(1-2y) = y\,(1-y)(1-2y) \qquad \text{(Eq. 4)}$$

Thus, y in function of s, gives a sigmoidal curve (Fig. 1) with an inflexion point for s=0 (i.e x=1) and for y=0.5 (see Eq. 4).

The advantage of Eq. 3 and 4 is that one can calculate very simply y' and y" as illustrated in Table I.

Since, as explained by Klotz (2) the inflexion point is a crucial test for being sure that at least half saturation (y=0.5) has been obtained, it becomes interesting to consider the values of y' in Table I.

They demonstrate that from a pure mathematical point of view the use of the above test is certainly correct. They show also how very thin is the margin allowed for the experimental errors in the Klotz graph. This assertion can be easily quantified by help of Eq. 3.

We have shown that

$$y' = \frac{e^s}{(1+e^s)^2} \quad \text{and} \quad y' = \frac{x}{(1+x)^2}$$

one can see that

$$\ln y' = \ln x + 2 \ln (1+x)$$

and therefore

$$\frac{dy'}{y'} = \frac{dx}{x} + 2.y\,\frac{dx}{x}$$

where dy' and dx are absolute errors.

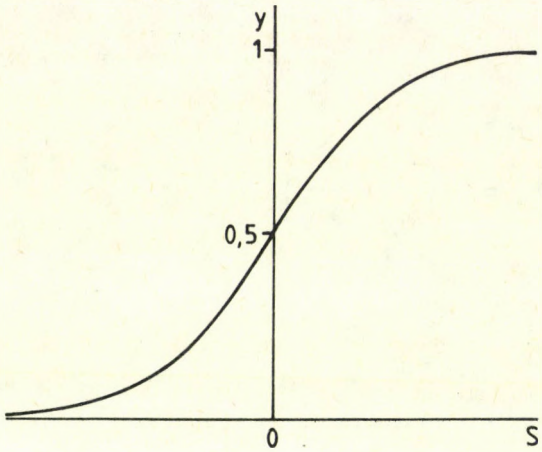


Figure 1 : The semi lagarithmic graph
S is the neperian logarithm of x.

TABLE 1 : Values of y' and y" in function of y

y' and y" are calculated from equations 3 and 4.
Note that the value of y' for $0.3 < y < 0.7$ quantify the subjective impression of a "linear" segment in Fig. 1.

y	y'	y"
0.1	0.09	0.072
0.2	0.160	0.096
0.3	0.210	0.084
0.4	0.240	0.048
0.5	0.250	0
0.6	0.240	-0.048
0.7	0.210	-0.084
0.8	0.160	-0.096
0.9	0.09	-0.072

The absolute error dy' is equal to

$$dy' = y' \left(\frac{dx}{x} + 2y^2 (1-y) \frac{dx}{x} \right)$$

Supposing that the relative error $\frac{dx}{x} = 0.05$ (5 %) one can calculate that for y = 0.3, $y' = 0.210 \pm 0.0168$; for y = 0.4, $y' = 0.240 \pm 0.0216$; for y = 0.5, $y' = 0.250 \pm 0.0250$; for y = 0.6, $y' = 0.240 \pm 0.0264$; for y = 0.7, $y' = 0.210 \pm 0.0252$.

In other words the errors concerning the slopes may render the determination of an inflexion point impossible between y = 0.3 and y = 0.7.

In fact the preceeding calculation does not take in account the error made on y which, in practice, is often measured independently from x. That can be done in the following way. (Note that eq. 1 gives y/x = 1-y (eq. 5) and y/x is depicted in Fig. 2 in function of y according to Scatchard).

Thus, one can see in Fig. 2 that the hached surface is equal to y'.

The relative error on this surface and thus on y' is equal to

$2 \frac{dy}{y} + \frac{dx}{x}$. If $\frac{dy}{y} = \frac{dx}{x} = 0.05$

the relative error on y' is thus $\pm$ 15 %, a result which again clearly shows that the finding of a inflexion point could be impossible if $0.3 \leqslant y \leqslant 0.7$. We conclude therefore that the "test of the inflexion point" lacks, at least, sensitivity. Therefore there is no reason for abandonning the Scatchard representation.

The limits of the semilogarithmic graph in the case of two independent but non equivalent systems of binding.

We will restrict this study to the case of results obtained with two independent binding systems out of which one is said "saturable" and the other one seems to remain "unsaturable". In practice this case is frequent. It is interesting therefore to apply the "test of the inflexion point".

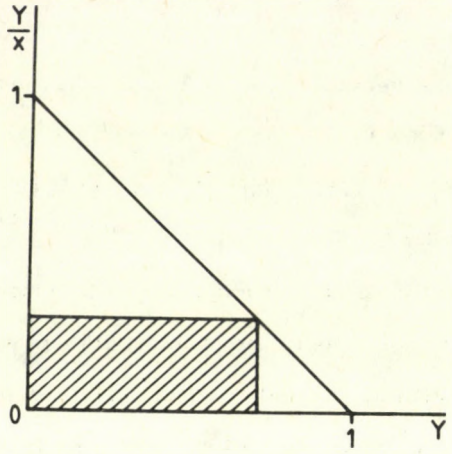


Figure 2 : The Scatchard plot
The hached surface is equal to y (1-y) = y' (see Eq. 3)

TABLE 2 : Values of y' in function of y in the competitive inhibition

(1) : a = 1 ; b = 1 (2) : a = 0.01 ; b = 1

y	y' (1)	y' (2)
0.1	0.091	0.065
0.2	0.155	0.103
0.3	0.202	0.133
0.4	0.229	0.156
0.5	0.237	0.171
0.6	0.228	0.174
0.7	0.200	0.163
0.8	0.154	0.134
0.9	0.088	0.082

The saturation fraction is to

$$y = \frac{e^s}{1 + e^s} + a\, e^s \qquad \text{(Eq. 6)}$$

$$\text{if } a = \frac{K_2 (P_T)_2}{K_1 (P_T)_1} \quad \text{and constant}$$

$(P_T)_1$ and $(P_T)_2$ are the total binding site - concentrations of the saturable and unsaturable systems respectively, whose K_1 and K_2 are the respective affinity constants for X, K_1 (X) = x = e^s, as before, and K_2 (X) $\ll$ 1.

The derivatives are

$$\frac{dy}{ds} = y' = \frac{e^s}{(1 + e^s)^2} + a\, e^s \qquad \text{(Eq. 7)}$$

$$\frac{d^2y}{ds^2} = y'' = e^s\,(2f^3 - f^2 + a) \qquad \text{(Eq. 8)}$$

$$\text{if } f = \frac{1}{1 + e^s}$$

Studying $g = 2f^3 - f^2$, one notes that

$$\frac{dg}{ds} = \frac{df}{ds} \cdot \frac{dg}{df} = \frac{-\, e^s}{(1+e^s)^2} \left[6f^2 - 2f \right]$$

Thus g has a minimum for f = 1/3 which corresponds to a value of

$$g = -\frac{1}{27}$$

The equation 8 becomes then

$$y'' = e^s \left[a - \frac{1}{27} \right]$$

This means that one can hope to see an inflexion point only if $a < 1/27$. Let us suppose that a = 0.05. Consequently the Klotz representation will never find out an inflexion point. However the Scatchard graph can be calculated following this equation.

$$\frac{y}{x} = \frac{1}{1+x} + 0.05 = 1 - y + 0.05$$

One can immediately see that when $x = 0$ ($y = 0$) $y/x = 1.05$ (instead to be equal to 1) and that when $x \longrightarrow \infty$ then $y/x \longrightarrow 0.05$. In this case the correction has to be that proposed by Rosenthal (7).

Moreover, even without this correction the Scatchard plot remains practically linear until $x/y = 0.25$. An extrapolation towards the abcisses yields $y = 1.35$ (instead of 1) which is an error nearer to the reality than that consequent of the Klotz analysis. As a matter of fact in this given case Klotz comes to the conclusion that, because of the absence of the inflexion point, half saturation can be never attained.

In conclusion we feel that the representation according to Klotz leads to erroneous interpretation of results obtained when working with crude extracts or even with purified proteins, because of a linearly increasing "background noise" due to the unavoidable imperfection of the techniques employed. Therefore it seems that the correction according to Rosenthal (7) is obligatory before trying to apply the test of the inflexion point.

General cases

Equation 2 can be written

$$y = \frac{ux}{1 + ux} = \frac{ue^s}{1 + ue^s} \qquad \text{(Eq. 9)}$$

where x and s are as before and u is an apparent affinity "constant" which in numerous cases varies with x (and so with s).

Then

$$\frac{dy}{ds} = y' = y\,(1-y)\,(1 + \frac{u'}{u}) \qquad \text{(Eq. 10)}$$

$$\frac{d^2y}{ds^2} = y'' = y\,(1-y)\,(1-2y)\,(1 + \frac{u'}{u}) + y\,(1-y)\,(1 + \frac{u'}{u})' \qquad \text{(Eq. 11)}$$

If u is constant, one finds again the equations 3 and 4. But if u, as indicated, is an affinity "constant" varying with e^S, is there then an inflexion point and does it occur for y = 0.5 ?

Let us suppose that it occurs for y = 0.5. Which is the form of u ? One can see that if y" = 0 for y = 0.5 then this implies that 1 + u'/u is constant and that u is an exponential of the type $u = a.e^{bs}$ where a and b are any two constants. In every other case if there is an inflexion point it will never occur for y = 0.5.

This might be valid for many arithmetic plots of velocity versus substrate concentration representing the Michaelis-Menten equation in the case of the enzyme reactions :

$$\frac{v}{V} = \frac{(S)}{Vm+(S)} = \frac{Km^{-1}\,(S)}{1+Km^{-1}(S)}$$

where v = initial rate, V = maximum reaction rate, (S) = substrate concentration and Km = Michaelis constant.

It is obvious that the Klotz representation can be readily used, as pointed out without any mathematical demonstration in a recent article (8).

If indeed we take

$$\frac{v}{V} = y,\ (S) = e^S \text{ and } Km^{-1} = u, \quad \text{where here, } s = \ln (S)$$

We find again $y = u.e^S/1+u.e^S$ and in the case of a Michaelis-Menten reaction (i.e Km remains constant) one can obviously calculate y' and y" as we did before. However we emphasize that u in enzymatic reactions different from the basic Michaelis-Menten case, can actually vary with (S) (or e^S). Thus the discussion we made before remains also true for enzymatic reactions.

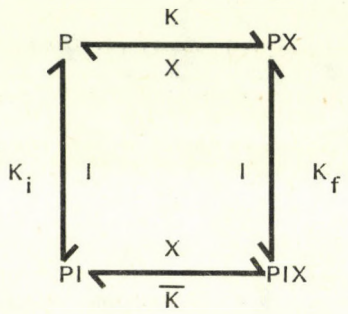
Coming back to the ligand binding problems, from equation 9, one can write that

$$\frac{y}{x} = u\ (1-y) \qquad \text{(Eq. 12)}$$

and that $\frac{y_o - y}{y_o} = (1-u)\ (1-y)$ (Eq. 13)

if $y_o = \frac{x}{1+x}$

In the general "square equilibrium" depicted by



where P is the binding site for X and I an effector (inhibitor or activator).

The apparent affinity "constant", u is equal to

$$u = \frac{1 + K_f(I)}{1 + K_i(I)} \qquad \text{(Eq. 14)}$$

where (I) is the concentration of a free effector.

Equation 13 becomes

$$\frac{yo - y}{yo} = \frac{K_i - K_f}{K_i}\ y_i \qquad \text{(Eq. 15)}$$

if $y_i = \frac{(PI)}{(P_T)}$

(see the square equilibrium (P_T) being the total binding sites i.e. equal to (P) + (PX) + (PI) + (PIX))

We note that I is an activator if (see eq. 13) $K_f > K_i$ or is an inhibitor if $K_i > K_f$.

In both cases Eq. 15 remains true.

If I is a competitive inhibitor ($K_f = 0$)

eq. 15 becomes $\frac{y_o - y}{y_o} = y_i$ (Eq. 16)

Limits of the semi-logarithmic representation in case of competitive inhibition.

Let us detail how to calculate y' in the important case of the competitive inhibition.

We now that, in this case

$$u = \frac{1}{1 + K_i\ (I)} = \frac{ax + by}{(1 + a)x + by} = \frac{ae^s + by}{(1+a)e^s + by} \quad \text{(Eq. 17)}.$$

when $a = \frac{1}{K_i\ (I_T)}$ and $b = \frac{(P_T)}{(I_T)}$

((I_T) is the total concentration of the competitive inhibitor, i.e. the sum of its free (I) and bound (PI) concentration). The value of u' is

$$u' = \frac{b\ (y' - y)}{(1+a)e^s + by}\ (1-u)$$

$$\text{thus } \frac{u'}{u} = \frac{1}{y} \cdot \frac{b\ (y' - y)\ (1 - u)}{b + a\ (e^s/y)} = \frac{1}{y} \cdot \frac{b\ (y' - y)\ (1-u)}{b + a\ (x/y)} \quad \text{(Eq. 18)}$$

It was demonstrated that

$$\frac{y_o}{y_o - y} = \frac{1}{y_i} = b + a\frac{x}{y}$$

a linear function which allows the measurement of K_i and (I_T) (9) if (P_T) is known.

Equation 18 becomes then

$$\frac{u'}{u} = \frac{1}{y} b\ (y' - y)\ (1 - u)\ y_i$$

When this value of u'/u is replaced in Eq. 10 one obtains that

$$y' = y\,(1-y) + b\,(y'-y)\,y_i^2 \quad \text{and} \quad y' = y\left(1 - \frac{y}{1 - by_i^2}\right) \qquad \text{(Eq. 19)}$$

y_i^2 in Eq. 19 can be calculated

$$\text{from } K_i\,(P_T) = \frac{y_i}{(1/b - y_i)\,(1 - y_i - y)}$$

for given values of a, b and y.

The y' values are so obtained from Eq. 19 are given in Table 2. This is an illustration of the discussion made precedently (see eq. 9) where u is varying with s. In this case, u varies with l which varies with s. Obviously the inflexion point test has its limits in the case of competitive inhibition chosen here as an example.

General Conclusions

1. Even in the simplest cases in ligand binding studies or in enzymology, errors of 5 % may lead already to misleading interpretations when using the Klotz-plot. This is obvious for the values y between 0.3 and 0.7.

2. The presence of an inflexion point even if one can show that it occurs for y = 0.5 does not yield univocal interpretation.

3. If one makes the slightest deviation from the simplest equations, the Klotz representation brings about complications in the interpretation without yielding clearcut information concerning the model of the reaction on deals with. This is true for the reactions having practically unavoidable background noise, and for cases when one would like to discriminate between a competitive or noncompetitive inhibitor.

Further, the Klotz graph requires a 2-stage interpretation. The first essential stage is the search for an inflexion point, and we have seen that this is far from being evident or simple. The second step is the search of the number of the sites (y = 1). (Evidently if the inflexion point has not been found there is no use to proceed to step 2).

Briefly, the representation according to Klotz is not a panacea : it is one of the several representations. For instance, Scatchard and Klotz graphs could be complementary as already pointed out (10).

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EFFECT OF METHOXY-p-BENZOQUINONES AND METHOXY-p-HYDROQUINONES ON DNA SYNTHESIS IN EHRLICH ASCITES TUMOR CELLS*

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The effects of various methoxy-p-quinones and methoxy-p-hydroquinones on DNA synthesis and thymidine transport in Ehrlich Ascites Tumor Cells was investigated. The inhibitory concentrations (IC_{50}) were in the range of 5 to 65 μM. Adriamycin, used as positive control, had an IC_{50} of 6 μM. 2,3-Dimethoxy-benzoquinone and -hydroquinone and 2,3,5,6-tetramethoxy-benzoquinone clearly inhibited DNA synthesis at concentrations where thymidine transport was not affected. 2-Methoxy-hydroquinone, 2-methoxy-benzoquinone, 2,6-dimethoxy-benzoquinone, 2,5-dimethoxy-benzoquinone had IC_{50}-values close to the dose inhibiting also thymidine transport. 2,5-Dimethoxy-benzoquinone, 2,3,5-trimethoxy-hydroquinone, 2,3,5-trimethoxy-benzoquinone and 2,3,5,6-tetramethoxy-hydroquinone strongly inhibited thymidine transport without a significant effect on DNA synthesis. Addition of ascorbate enhanced the inhibitory activity of 2,6-dimethoxy-benzoquinone.

INTRODUCTION

The quinone group is present in many biologically active compounds such as ubiquinones, plastoquinones, menadione, vitamine K, anthracyclins and mytomycins. Moreover quinoid compounds are formed in vivo by biotransfor-

Abbreviations: EATC, Ehrlich Ascites Tumor Cells; ^{3}H-dT, methyl-^{3}H-deoxythymidine; HBSS, Hank's balanced salt solution

* *In memoriam Albert Szent-Györgyi*

mation from many phenolic compounds. The best known example of which is dopaquinone derived from tyrosine. The biological activity of quinones has been related to their ability to take part in redox cycling and generate reactive free radicals (1-3) and to their potential to arylate nucleophilic compounds, in partcular those containing SH-groups (4,5). In case of the more complex antitumor agents adriamycin, daunorubicin, mitomycin C and aziridinylbenzoquinone, intercalation with DNA, alkylation of DNA bases and membrane effects may also contribute to the specific interaction with tumor cells and their cytotoxicity (reviewed in ref. 3). The simple benzoquinone is a potent antimitotic compound and its ability to inhibit cell growth was related to blockage of essential SH-groups (5,6). 2-Methoxy-benzoquinone and 2,6-dimethoxy-benzoquinone are constituents of the wheat germ (7) and the later compound was also reported to be the active principle of extracts from some tropic plants active against the transplanted tumor P-388 (8). It was also shown (9,10) that mixtures of ascorbic acid with 2,6-dimethoxy-p-benzoquinone inhibit growth of EATC in vivo and, since ESR measurements showed that the mixture generated a rather long-lived population of reactive free radicals, it was assumed that these radicals are in some way involved in the inhibition of tumor cell growth. The increased frequency of certain tumors among workers in the wood industry was suspected to be due to some wood constituents, such as 2,6-dimethoxy-benzoquinone (11), however in the Ames test this quinone shows only a very weak mutagenicity. In this investigation the effect of various methoxy-benzoquinones and methoxy-hydroquinones on the DNA synthesis of EATC are described.

MATERIALS AND METHODS

The various methoxyquinones and hydroquinones were synthetized by one of us(G. Fodor) for various biological studies carried out by A. Szent-

Györgyi's group at the Laboratory of the National Foundation for Cancer Research at the Marine Biological Laboratory, Woods Hole, MA. Prof. Albert Szent-Györgyi was highly interested in the mechansim of the cytotoxic action of quinones (12) and gave these compounds as a generous gift to H. Esterbauer. Adriamycin was purchased from Sigma; |methyl-^{3}H|-deoxy-thymidine was from Amersham and had a specific activity of 5 µCi/mmol. The cocktail for radioactivity counting was Beckman MP. All other chemicals were either from Merck or from Sigma.

The thymidine incorporation assay was carried out essentially as described by others (13,14). The only important modification was that both the radioactivity present in the TCA-insoluble part and in the soluble fraction were measured. This allowed to calculate the overall cellular uptake of ^{3}H-dT and its disturbance by the drugs. 10×10^6 EATC were injected in the peritoneum of male NMRI-mice. On the 6th to 11th day the mice were put to death by fracture of the neck and the ascites fluid was taken from the open peritoneum. 1000 IU Heparin per mouse were added to the cell suspension and the cell concentration was measured by hematocrit. HBSS was added to 400×10^6 EATC to give a final volume of 50 ml. The suspension was preincubated for 2 hours in an open glass baker on a water bath at 37° C and gently stirred with a teflon twirling-stick. Thereafter, 1 ml of the resulting cell suspension was transferred to a 20 ml glass vial (liquid scintillation counting vial) and mixed with 3 ml of HBSS containing the compound to be investigated. Each assay was made in triplicate. The vials were incubated for 20 min at 37° C on a shaking water bath at approximately 40 rpm. 1 µCi of ^{3}H-dT dissolved in 0.1 ml HBSS was then added to each vial and the incubation was continued for 30 min under the same conditions. Thereafter, the cells were collected by centrifugation, washed once with 5 ml ice-cold 0.9% NaCl and precipitated with 5 ml ice-cold 5% TCA. After 30 min on ice, the suspension was centrifuged. The clear supernatant was decanted into a 20 ml vial and evaporated over night in a drying oven at 80° C. The remaining liquid (about 1-2 ml) was mixed with 10 ml cocktail and the radioactivity was counted by a Beckman 4800 instrument to an accuracy of 2%. All cpm values were quench-corrected to obtain the dpm-values. The data of this measurement give the amount of ^{3}H-dT taken up by the cells but not incorporated into DNA.

The centrifugation-pellet was mixed with 0.25 ml of 1N HCl and heated 15 min on a boiling water bath. Thereafter 0.5 ml 1N NaOH were added and heating on the boiling water bath was continued for additional 15 min. Finally the solution was neutralised by 0.25 ml of a mixture from acetic acid : Triton X 100 : water (2:1:7;v/v/v), vortexed for 10 sec, diluted with 10 ml cocktail and counted for radioactivity to an accurancy of 2%, the cpm data were quench corrected to obtain the dpm values. This measurement gives the amount of ^{3}H-dT which was incorporated into the DNA.

RESULTS

Fig. 1 shows the kinetics of a representative thymidine incorporation experiment with EATC in the absence of an inhibitor. The amount of ^{3}H-dT incorporated into the DNA (curve A) increased nearly proportionally with the incubation time, whereas the thymidine pool, i.e thymidine in the cells yet not incorporated into DNA (curve B) reached a maximum after

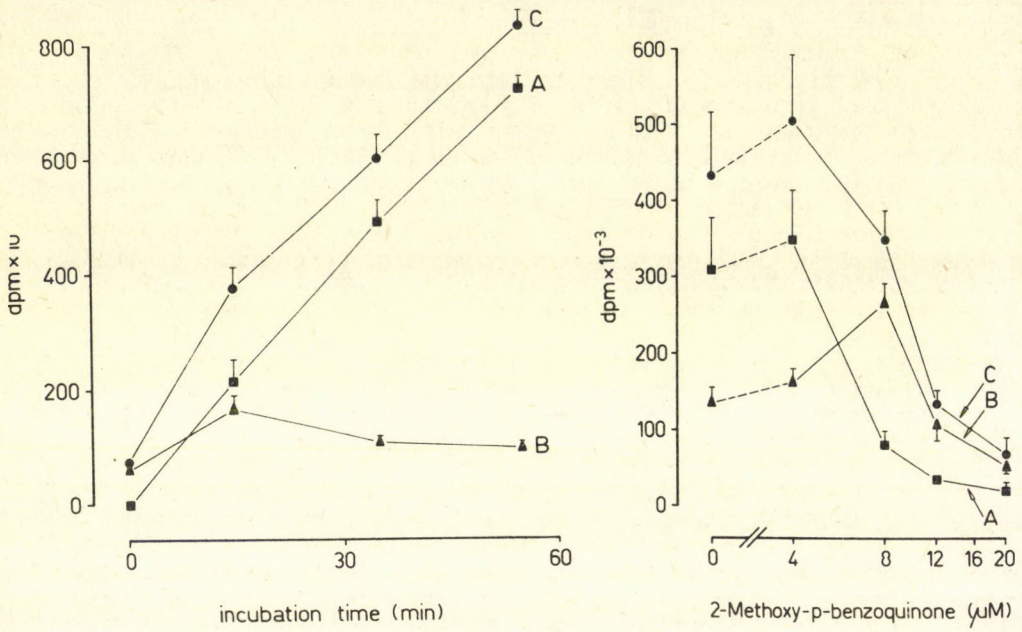

Figure 1:
Kinetics of the thymidine incorporation into DNA and total thymidine uptake by EATC in the absence of an inhibitor

8×10^6 EATC in 4 ml HBSS were incubated for various times with 1µCi ^{3}H-dT, thereafter the thymidine not taken up by the cells was removed and the radioactivity present in the TCA insoluble fraction (curve A) and TCA soluble fraction (curve B) was measured. Curve C is the sum of radioactivity in A and B and corresponds the total ^{3}H-dT taken up by the cells. The bars give the standard deviation from 3 experiments.

Figure 2:
Dose-effect curve of 2-methoxy-p-benzoquinone

8×10^6 EATC in 4 ml HBSS were incubated in the presence of 0 (=control), 4, 8, 12, and 20 µM of the quinone and 1 mCi ^{3}H-dT. After 30 min excess thymidine was removed and the radioactivity present in the TCA insoluble fraction (curve A) and TCA soluble fraction (curve B) was measured. C is the sum of radioactivity in A and B and corresponds the total ^{3}H-dT taken up by the cells. The bars give the standard deviation from 3 experiments.

about 15 min and thereafter slowly decreased. The sum of both (curve C) can be taken as direct measure for the thymidine transport capacity of the cells. After 55 min 36.6% of the initial offered radioactivity was cell associated, from that 88% were TCA insoluble and therefore incorporated into the DNA and the remaining 12% were TCA soluble . This distribution showed some variation with different experiments but in all cases the fraction bound to DNA was at least 70% of the total radioactivity taken up by the cells. In additional experiments not reported here, it was ascertained that the washing procedure used removes 98% of the ^{3}H-dT not taken up by the cells and that no radioactivity is released by the cells back into the washing medium.

It is clear that any disturbance of the thymidine transport or its incorporation into DNA by a drug must result in an altered distribution in the cell. Fig. 2 shows as example the dose-effect curve of 2-methoxy-benzoquinone on thymidine uptake and incorporation into DNA. At 8 µM the incorporation into DNA is reduced to 22% of the uninhibited control, whereas the free ^{3}H-dT not bound in DNA is significantly higher relative to the control. The total thymidine taken up is less affected. This clearly indicates that 8 µM of 2-methoxy-benzoquinone inhibits the incorporation of thymidine into DNA without affecting the transport of thymidine across the cell membrane. With higher concentrations this specific effect is gradually reduced and the transport of thymidine is also inhibited as evident by the rapid decrease of the total thymidine taken up. Another type of inhibition is produced by 2,5-dimethoxy-hydroquinone as illustrated in Fig. 3. A dose of 20 µM decreases both the overall uptake and the DNA incorporation to about the same extent (64-67% of the control), whereas the cellular free thymidine content is reduced only to 76% of the control. These results strongly suggest that the predominant effect of the hydro-

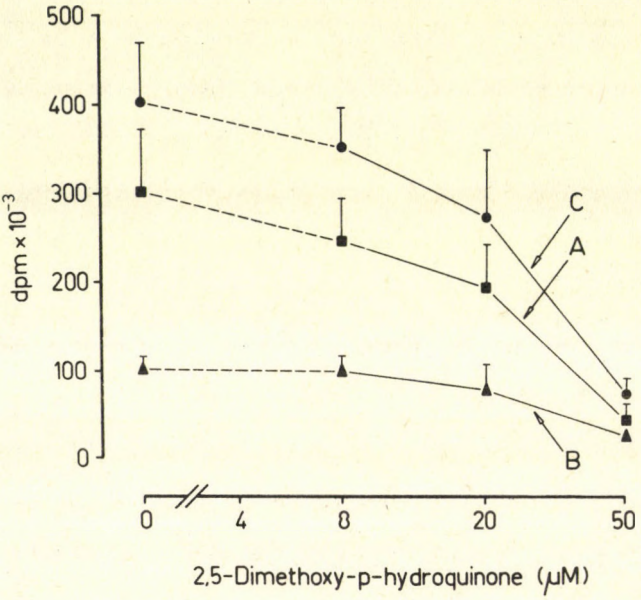

Figure 3:
Dose-effect curve of 2,5-dimethoxy-p-hydroquinone
Experimental conditions and symbols are as in fig. 2.

quinone is the inhibition of the thymidine transport into the EATC. A third type of inhibition is possible when both uptake and incorporation are inhibited at about the same concentration and in this case it is not possible to determine which effect is predominant.

Twelve methoxy-substituted quinones and -hydroquinones, respectively, were investigated in respect to their inhibitory activity on ^{3}H-dT uptake and incorporation into DNA as shown in Fig. 2 and 3. Table 1 depicts the dosis leading to 50% inhibition of the incorporation of ^{3}H-dT into the DNA (IC_{50}) as well as the inhibition of the total thymidine uptake at the IC_{50}, compared to the control.

Several quinones were also tested in the presence of a 10-fold excess of ascorbic acid, since it has been reported (9,10), that this mixture is by far more active than the quinone alone in inhibiting growth of EATC. With 2-methoxy-benzoquinone and 2-methoxy-hydroquinone ascorbic acid addition was ineffective, whereas in the case of 2,6-Dimethoxy-benzoquinone ascorbate clearly enhanced the quinone effect. Ascorbate alone did not affect thymidine uptake and incorporation up to concentrations of 1000 µM. Adriamycin, a quinone anti-cancer-drug, with proven inhibitory effect on DNA synthesis (3), was also tested as positive control.

DISCUSSION

The assay developed and employed in this study includes measurements of thymidine incorporated into the DNA (TCA-insoluble fraction) and total ^{3}H-dT uptake by the cells (TCA insoluble + TCA soluble fraction) and the dependence of these two parameters from the concentrations of the inhibitory compounds. The advantage over the conventional assay measuring the radioactivity in the TCA-insoluble fraction only, is that the dose effect curves thus obtained allow to some degree to conclude if the drug in fact

TABLE I
Effects of methoxy-quinones and methoxy-hydroquinones on the incorporation of thymidine into DNA of EATC and on the total uptake of thymidine by the cells.
The doses leading to a 50% inhibition of the incorporation of ^{3}H-dT into DNA (IC_{50}) were determined by interpolation from the dose effect curves, similarily the inhibition of the total ^{3}H-dT uptake was estimated. Reported are the single values found at IC_{50} as percentage of the uninhibited control. The IC_{50} values are the means ± S.D. from 3 to 6 experiments (in brackets) with different EATC preparations.

System	IC_{50} (μM)	total ^{3}H-dT uptake at IC_{50} in % of control
2-methoxy-benzoquinone[b]	9.2 ± 5.0 (5)	88, 61
2-methoxy-benzoquinone+ascorbate[b]	5.9 ± 0.4 (3)	
2-methoxy-hydroquinone[a]	9.9 ± 3.3 (6)	70, 53, 78
2-methoxy-hydroquinone+ascorbate[b]	9.0 ± 0.4 (3)	
2,3-dimethoxy-benzoquinone[a]	6.3 ± 0.8 (3)	76, 71
2,3-dimethoxy-hydroquinone[a]	8.0 ± 1.6 (4)	72, 59, 79
2,5-dimethoxy-benzoquinone[c]	23.4 ±11.8 (3)	54, 50
2,5-dimethoxy-hydroquinone[c]	23.2 ±12.5 (3)	53, 55
2,6-dimethoxy-benzoquinone[b]	11.8 ± 2.0 (5)	51, 61, 67
2,6-dimethoxy-benzoquinone+ascorbate[b]	5.7 ± 1.6 (3)	49, 61
2,3,6-trimethoxy-benzoquinone[c]	32.8 ± 5.7 (3)	62, 53
2,3,6-trimethoxy-hydroquinone[c]	15.5 ± 0.9 (3)	54, 55
2,3,5,6-tetramethoxy-benzoquinone[a]	51.6 ± 2.8 (3)	62, 62
2,3,5,6-tetramethox-hydroquinone[c]	17.4 ± 3.7 (3)	53, 57
2,6-dimethoxy-3-(n-butoxy)-benzoquinone[b]	29.8 ± 8.3 (3)	59, 65
adriamycin[a]	5.7 ± 1.3 (3)	67, 72

a,b,c: evidence for inhibition of de novo synthesis of DNA
a: strong evidence, b: less strong evidence, c: no evidence

inhibits the de novo synthesis of DNA or if the reduced incorporation of radioactivity into the DNA could also result from an inhibition of the thymidine transport. All quinones and hydroquinones tested strongly reduced the incorporation of ^{3}H-dT into the DNA of EATC in the micromolar range. Most effective in this respect were 2-methoxy-benzoquinone, 2-methoxy-hydroquinone, 2,3-dimethoxy-benzoquinone, 2,3-dimethoxy-hydroquinone and 2,6-dimethoxy-benzoquinone which showed IC_{50} values comparable to that of adriamycin. From the total uptake of ^{3}H-dT relative to the control it became evident that all substances listed in Table 1 can also block the ^{3}H-dT transport if used at concentrations 3 to 5 times higher than the IC_{50}. At lower concentrations some of the quinones (i.e 2,3-dimethoxy-hydroquinone; 2,3-dimethoxy-benzoquinone; 2,3,5,6-tetramethoxy-benzoquinone and 2,3-dimethoxy-5(n-butoxy)-benzoquinone inhibited the incorporation of ^{3}H-dT into DNA without significantly affecting the thymidine transport, which strongly indicates a direct effect of these substances on the de novo synthesis of DNA. 2-Methoxy-hydroquinone, 2-methoxy-benzoquinone and 2,5-dimethoxy-hydroquinone inhibited incorporation of ^{3}H-dT into DNA synthesis and ^{3}H-dT transport at about the same concentrations, whereas all other substances preferentially affect the thymidine transport at the IC_{50} dose. Adriamycin was, as expected, a strong inhibitor for DNA synthesis with an IC_{50} of 5.7 µM. But at higher concentrations of 20 µM adriamycin also blocked ^{3}H-dT transport as evident from the decreased (50% of control) total ^{3}H-dT uptake.

Two mechanisms for cytotoxic effects of quinones are discussed in the literature, formation of reactive oxygen radicals through redox cycling (1-3,15) and arylation of functional SH-groups (4,6,11). The finding that the hydroquinones show similar activities as the corresponding quinones is in favour of the redox cycling hypothesis. This is also supported by the

inhibitory effect of 2,3,5,6-tetramethoxy-benzoquinone, since this compound has no electrophilic center for nucleophilic attack of SH-groups. The results described here might have some biological implications since they show that simple quinones and their reduced counterparts are able to affect both membran functions, as measured by the ^{3}H-dT transport, and the de novo synthesis of DNA in the micromolar concentration range. Quinones are widely distributed in the nature and some of them as 2,6-dimethoxy derivatives are part of the human diet and their high biological activity as reported here deserves further attention.

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THE EFFECT OF WATER AND IONS ON THE ENERGY BAND STRUCTURE OF PERIODIC α-HELICAL POLYPEPTIDES*

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The solvent structure around several periodic polypeptides in the α-helical conformation has been determined by Monte-Carlo simulations. The calculations of the water clusters have been performed in the presence and absence of Na^+ ions. The resulting hydration shell has been used to calculate the band structure of the polymers in the effective field of the water molecules and ions. The results show that the maximum change of the band widths and the band gaps is about 0.7 eV in the presence of the hydration shell. The band positions are shifted by up to 4.1 eV in the presence of water molecules and ions. The results have been compared with the band structure results of periodic polypeptides in the β-pleated sheet conformation.

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INTRODUCTION

In recent years the effect of environment on the electronic structure of a biopolymer has been taken into account [1,2] and the Monte Carlo method has been used already many times to determine the water structure around biomolecules [3-7]. In two previous papers [8,9] standard Monte Carlo experiments have been performed for studying the solvent structure around ß-pleated sheet polypeptides (ß-polypeptides). The hydration shell obtained in this way has been used to investigate its effect on the band structure of ß-polypeptides. Since the conformations of many polypeptides is an α-helix, in this paper the solvent structure around several periodic polypeptides in the α-helix conformation (afterwards abbreviated as α-polypeptides) has been determined by Monte Carlo simulations. The resulting hydration shells have been used to calculate the band structures of α-polypeptides in the effective field of the surrounding water molecules.

One of the motivations for this work was to study the effect of water and ions on the electronic structure of α-polypeptides. Another goal was to compare the electronic band structure of α-polypeptides with that of ß-polypeptides.

METHODS

The method involves three steps. Monte Carlo simulation, calculation of the effective potential of the water molecules and ions which act on the polypeptide and crystal orbital band structure calculation. The first step is to determine the positions of the water molecules and ions surrounding the α-polypeptide. The second step is to find a suitable representation of the electron distribution of the water cluster (with ions or without them) by point charges. The third step is to obtain the electronic structure of the α-polypeptides in the effective field of the surrounding water molecules and ions.

The Monte Carlo simulation was performed for a solution-density of 1.0 g/cm^3. In the beginning the system has been

heated up to 3000°K for 128 cycles (the system without ions) or 2048 cycles (the system with sodium ions) to eliminate the influence of the starting positions, then cooled down to 300°K and held at this temperature for the rest of the simulation.

Since in an α-helix there are 3.6 amino acid residues in one turn of the helix the minimum sample box for strict periodic boundary conditions has to include 18 amino acid residues. Such a system is too large for Monte Carlo simulation. In this work periodic boundary conditions for the complex (translation and rotation) helix operations have been used. That means if the position of a water molecule or sodium ion is outside the boundaries of the sample box, it will be placed to the corresponding point according to the translation vector and rotation angle (for one amino acid residue in the sample box) or their multiple (for more than one amino acid residues in the sample box). We have developed a general program for the computer simulation of the solvent structure of biomolecules with helical symmetry like α-polypeptides. In this work a rectangular parallelepiped containing four amino acid residues has been chosen as sample box. The height of the box is, therefore four times the translation distance of an α-polypeptide; the length and width are determined by the program, so as to include at least two solvation shells and part of the bulk water.

The amino acid residues and water molecules contained in the box were all regarded as rigid. The geometry of a water molecule was taken from the results of Benedict et al. [10]. For α-polypeptides a right-handed helix ($\phi = 123^{\circ}, \psi = 135^{\circ}$) of L-amino acids was used [11].

The water-water interaction potential used here is the approximate analytical solution fitted to limited CI calculations by Clementi et al. [12].

The amino acid water, amino acid Na^+, Na^+-Na^+ and water-Na^+ interaction potentials are also approximate analytical solutions fitted to calculations by Clementi et al. [13-15].

The Monte Carlo Metropolis sampling scheme has been adopted [16]. The position and orientation of the water molecules were determined by six random variables ($x, y, z, \Theta, \phi, \gamma$

and for sodium ions by three random variables (x,y,z). The configurations corresponding to the minimum total energy has been chosen for the water cluster (with sodium ions or without them) [8].

The final point charge coordinates of the water molecules in the solvation shell are obtained by representing each water molecule by an array of 23 point charges as described in ref. [17].

For keeping electric neutrality negative point charges have been put at suitable positions of the system if it contained sodium ions. The positions of the negative point charges were chosen such that they were compatible with the water structure; namely they were positioned at a distance of 3.5 - 4.0 Å from the corresponding Na^+ ion in a direction away from the polymer with small deviations such that a place was found where the surrounding water molecules pointed with the positive ends towards the negative point charge. The potential of the point charges describing the electrons and nuclei of the water molecules, of the Na^+ ions and of the negative countercharges were added to the one-electron part of the Fock operator. The crystal orbitals were determined then with the help of this modified operator F. In this work each water molecule has been represented by 23 point charges, and each sodium ion by 1 point charge.

All band structure calculations have been performed with the help of the ab initio crystal orbital method [18,19], using Clementi's 7s3p/2s1p (for C,N and O) and 4s/1s (for H) minimal basis sets [20].

Since there are 3.6 amino acid residues in each turn of an α-polypeptide and we want to cover at least one turn (to include also the inter-ring interaction), the first neighbors's interaction approximation has been used, the elementary cell containing two amino acid residues in the elementary cell.

All calculations have been performed on the IBM 3090 computer and on FPS processors in a single execution mode at the IECSEC of IBM Italy in Rome.

RESULTS

In this work we have selected four model systems: α-polyglycine with water (α-Gly+W), α-polyglycine with water and ions (α-Gly+W,I), α-polyalanine with water (α-Ala+W), α-polyalanine with water and ions (α-Ala+W,I). For the Monte Carlo simulation we have calculated the total interaction energy of the system in 10240 cycles (one cycle implies a move of each molecule separately and successively). The first 5120 cycles towards the energy minimum were discarded from the statistical analysis. The following results are based on the averaging over the last 5120 cylces. As a rule of thumb we chose the step length for one move of a particle in such a way that about half of the random configurations were acceptable [21].

Table 1: Interaction Energies (in kcal/mol) for Monte Carlo Results for α- and β-polyglycine and polyalanine [9]

System	β-poly-ala with water	β-poly-ala with water, $Na^+(4)$	β-poly-ala with water, $Na^+(2)$	β-poly-gly with water	α-poly-ala with water	α-poly-ala with water $Na^+(4)$	α-poly-gly with water
E_{WW}	-8.90	-4.45	-6.9	-9.0	-8.6	-3.5	-8.9
E_{WA}	-6.4	2.14	-1.6	-13.0	-19.7	0.55	-19.5
E_{II}		12.7	0.1			91.0	
E_{IA}		-54.1	-29.5			-26.6	
E_{WI}		-85.7	-66.1			-113.0	

In Table 1 the different interaction energies (in kcal/mol) between water-water$^{(E_{WW})}$, amino acid-water$^{(E_{WA})}$, Na^+-$Na^{+(E_{II})}$, Na^+-water$^{(E_{IW})}$ and amino acid-$Na^{+(E_{IA})}$ are listed together with the corresponding results in the case of β-polypeptides [9] for comparison. E_{WW} is the average of the interaction energies between one water molecule and the other water molecules. E_{WA} is the average of the interaction energies

between the four amino acid residues (for α-polypeptides, two residues for β-polypeptides) and the water molecules in the box. E_{II} is the average of the interaction energies between one sodium ion and the other sodium ions within the box. E_{IA} is the average interaction energy between the four amino acid units (for α-polypeptides, two units for β-polypeptides) and the sodium ions in the sample box. E_{WI} is the average of E_{WI} and E_{IW}, where E_{WI} is the average of the interaction energies between one water molecule and the sodium ions in the sample box and E_{IW} is the average interaction energy between one sodium ion and the water molecules in the sample box. For the sake of comparison with other Monte Carlo calculations they are divided by two, contrary to the bonding energies.

A. Systems in the presence of water (α-Gly+W and α-Ala+W)

The results show that the water-water interaction energy for α- and β-polypeptides are equal. The water-amino acid interaction energy, however, is much less in the case of β-polypeptides than for α-polypeptides. The reason is that due to the different conformations of α- and β-polypeptide chains the number of α-amino acid residues in the cube is larger than the number of β-amino acid residues.

In the α-Gly+W system four water molecules have been found which interact strongly with the glycine residues (the interaction energy is larger than -5.0 kcal/mol) and in the α-Ala+W system there are five water molecules. One can consider these water molecules as bonded ones.

B. Systems in the presence of water molecules and ions (α-Gly+W,I; α-Ala+W,I)

The results show that the water-water interaction energy is much less in the case of α-polyalanine with sodium ions than for α-polyalanine without sodium ions. The α-alanine-water interaction energy becomes positive. The number of bound water molecules is reduced to one. For the α-Gly+W,I system the results are similar that means that the water-water and amino-

acid-water interactions are much weakened by the presence of sodium ions. The reason is that due to the very strong Na^+-water and Na^+-amino acid interactions the position and orientation of the water molecules are strongly changed. These results are very similar to those obtained for ß-polypeptides with water molecules and ions.

Table 2: Band Structure Results for Polypeptides in the Presence of Water and Na^+ Ions (all energies in eV)

System[a]	Type of band[b]	Lower Limit	Upper Limit	Band Width	Band Gap
α-polyala	N+1	8.214	3.641	0.400	14.762
F	N	-12.026	-11.548	0.478	
α-polyala	N+1	2.714	3.104	0.390	14.619
W	N	-12.367	-11.905	0.462	
α-polyala	N+1	0.132	0.515	0.383	14.112
W,I	N	-14.135	-13.980	0.155	
α-polygly	N+1	3.056	3.463	0.407	14.815
F	N	-12.214	-11.760	0.454	
α-polygly	N+1	0.654	1.044	0.390	14.501
W	N	-14.312	-13.847	0.465	
α-polygly	N+1	-1.445	-1.202	0.243	14.392
W,I	N	-16.058	-15.837	0.221	

a) The subscript F means free-polymer calculations, W means calculation in the presence of water, W,I means calculation in the presence of water molecules and Na^+ ions.

b) N stands for the valence, N+1 for the conduction band, respectively.

In Table 2 we list the band edges, band widths and band gaps for α-glycine and α-alanine with water or with water and ions. The results show that:

1. In comparison with the free polymer the band gaps of the other systems are somewhat decreased by the presence of solvating water molecules and ions. For the systems with only

water (α-gly+W, α-ala+W) the band gaps are decreased by 0.31 eV and 0.14 eV, respectively, and for the systems with water and ions (α-gly+W,I, α-ala+W,I) the gap-values are decreased by 0.42 eV and 0.65 eV, respectively. The results indicate that the influences of water and ions on the energy band structures of periodic α-polypeptide are cooperative. The possible reason of this is that one of the sodium ions is in the middle of the α-helix, so that the interaction between the ions and the peptide is direct (i.e. it is not screened by water molecules positioned between them) and furthermore the polarization effect of the ions on the water molecules pulls them nearer to the peptide in this case. The opposite behaviour is found for many β-polypeptides, e.g. β-polyalanine in a model calculation comparable to the present one [9] because in that case both ions are polarizing the water molecules away from the peptide group, thus the polarization effects of the ions and the peptide on the water molecules cancel each other to some extent: they are anticooperative.

2. For all of the systems both valence and conduction bands are shifted down by the surrounding point charges. The shifts in the α-polyglycine with point charges is larger than those in α-polyalanine with point charges. The reason, as has been discussed in the previous paper [9] can be found in the local orientation and position of the water molecules which are nearest to the amino acid.

3. The band widths in the presence of water are almost unchanged. The differences of the valence band widths for α-polygly and α-polyala are 0.011 eV and 0.016 eV, respectively, the differences of the conduction band width 0.017 eV and 0.010 eV, respectively. In comparison with the free polymer the band widths are only little decreased by the presence of water and ions. The differences of the valence band widths for α-polygly and α-polyala are 0.23 eV and 0.32 eV, respectively, the differences of the conduction band widths 0.16 eV and 0.02 eV, respectively.

The above results show that the water and ions will slightly descrease the band gaps of α-polypeptides. Since the band gaps of the free polypeptide are large in our calculations (about 15 eV), the effects of water and ions on the band gap and, therefore, on the conduction properties of the α-polypeptides are rather weak.

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ISOLATION OF GLUCOSIDES OF METHOXYHYDROQUINONES FROM WHEAT GERM*

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Glucosides of methoxy-substituted hydroquinones were isolated from wheat germ and purified by displacement chromatography. The three major components obtained in pure form were identified by ^{1}H-NMR and mass spectrometry as (3-methoxy-4-hydroxyphenyl)-β-glucopyranoside, (3-methoxy-4-hydroxyphenyl)-β-cellobioside and (3-methoxy-4-hydroxyphenyl)-β-cellotrioside. HPLC was employed for the quantitative analysis of these compounds and their degradation products. Acid hydrolysis and oxidation yielded 2-methoxybenzoquinone and 2,6-dimethoxybenzoquinone from the glucosides present in wheat germ at levels of 3.5 and 1.0 micromoles per gram. The glucosides in various fractions of wheat germ extract were assayed by conversion to quinones and concomitant HPLC analysis.

INTRODUCTION

The two methoxysubstituted benzoquinones, 2-methoxybenzoquinone (2-MBQ) and 2,6-dimethoxybenzoquinone (2,6-DMBQ), have been isolated from a variety of plants and 2,6-DMBQ has been found in over 47 genera in 28 families (1). One of the largest natural sources known for these compounds is wheat (Triticum vulgaris), where derivatives of 2-MBQ and 2,6-DMBQ are located primarily in the germ (2). Vuataz (3), when attempting to determine the role of wheat germ in the poor rising of dough, extracted both 2-MBQ and 2,6-DMBQ from the germ after incubation with yeast and potassium bromate. However, he wrongly identified 2-MBQ as furfuryl glyoxal and the correct identification was made by Cosgrove et al. (4). It was found by DeJong et al. (5, 6) that yeast was not required for obtaining 2-MBQ and 2,6-DMBQ, and they postulated that the quinones are pre-

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sent in wheat germ as glucosides that are hydrolyzed by a glucosidase.

Recently, Graveland et al. (7), in the study of glutenin, isolated from wheat flour a component that was capable of solubilizing proteins that were insoluble in sodium dodecylsulfate and identified it as a trisaccharide of 2-methoxyhydroquinone (2-MHQ). Olsen (8) has shown that oat seedlings also contain a glucoside of 2-MHQ which could be readily hydrolyzed and oxidized to the corresponding benzoquinone.

The methoxybenzoquinones are highly reactive and known to potent inhibitors of fungi (9). Both 2-MBQ and 2,6-DMBQ inhibit bacterial growth by reacting with sulfhydryl or amino compounds (5, 10). On the other hand, the corresponding glucosides have not been found to be active. The glucosides may be storage forms of the quinones since infection of plants with fungi or bacteria is known to trigger an increase in glucosidase activity (8). Alternatively, the formation of glucosides from the quinones may be a means for inactivation of toxic by-products (11).

Recently, Szent-Györgyi suggested that 2-MBQ and 2,6-DMBQ may play a role in a hitherto undiscovered secondary metabolic pathway in animals (12). He postulated that the two compounds take part in a series of redox reactions that also involve ascorbic acid.

This study was undertaken to develop a procedure by using novel chromatographic techniques to isolate quinone derivatives from wheat germ for eventual investigation of their possible biological role in view of Szent-Györgyi's hypothesis.

MATERIALS

Wheat germ was a donation from International Multifoods (New Hope,MN). 2-MBQ, 2-MHQ, 2,6-DMBQ and 2,6-DMHQ were gifts from Dr. G. Fodor (University of West Virginia). HPLC grade methanol, acetonitrile, phosphoric acid, and sodium phosphate, as well as reagent grade cupric nitrate were obtained from Fisher (Fair Lawn, NJ). Glutathione was obtained from Sigma (St. Louis, MO). Hydrogen peroxide (3%) was purchased from Mallinkrodt (Paris, KY). Amberlite XAD-2 and IRC-718 resins were acquired from Rohm and Haas (Philadelphia, PA). Amberlite XAD-2 was ground and screened to obtain a 44-63um fraction, which was packed into a stainless steel column (250x16 mm).

Amberlite IRC-718 resin was treated with $Cu(NO_3)_2$ and used in the copper form. HPLC columns (250x4.6 mm) with 5 micron octadecyl-silica were from IBM Instruments (Danbury, CT) and columns (150x4.6 mm) home-packed with 10 micron octadecyl-silica from Amicon Corp. (Danvers, MA) were also used.

INSTRUMENTATION

The analytical liquid chromatograph consisted of a Perkin Elmer (Norwalk, CT) Series 10 pump, Rheodyne (Cotati, CA) Model 7010 injection valve with 20 microliter loop, Kratos (Ramsey, NJ) Model 773 variable wavelength UV detector and Bioanalytical Systems (West Lafayette, IN) Model LC-4B amperometric detector with dual cell. Chromatograms were obtained on a Kipp and Zonen (Delft, The Netherlands) Model BD-41 chart recorder.

Displacement chromatography was carried out as described elsewhere (13) by using a Perkin Elmer Series 10 pump and a Rheodyne 7010 sampling valve with 10 ml loop. A SSI (State College, PA) three-way valve was employed to switch from carrier to displacer by the stop-flow technique and a Knauer (Berlin, West Germany) differential refractometer was used to monitor the effluent.

The products were analyzed at the Yale Chemical Instrument Center by FAB-mass spectrometry using a Kratos MS-80 mass spectrometer, as well as by ^{1}H-NMR using a Bruker 500 MHz NMR instrument.

METHODS

Quinone formation. In a typical experiment 1 ml aliquots of the extract were mixed with 1 ml 3% hydrogen peroxide, 0.1 ml 50 mM $Cu(NO_3)_2$ and 7.9 ml of 50 mM NaH_2PO_4, pH 2.5. The mixture was heated at 100°C for two minutes and filtered through a 0.45 micron Millipore membrane.

HPLC analysis. The mobile phase was 50 mM NaH_2PO_4, pH 6.0, containing either 4% or 15% (v/v) acetonitrile for the analysis of the conjugates and the quinones, respectively. The flow rate was 1.0 ml/min. The column effluent was monitored with the amperometric detector and UV detector in series. The applied voltages of the dual amperometric detector were -200 mV and +800 mV and the UV detector was set at 210 nm.

Isolation of the glucosides. 500 g of defatted wheat germ were added to 1250 ml distilled water at 4°C. The suspension was sparged with nitrogen, periodically stirred for 25 minutes, and then centrifuged in two batches at 5000 rpm for 60 minutes. The combined pellets were extracted a second time with 500 ml of water. The supernatants were combined, mixed thoroughly with an equal volume of acetonitrile, and centrifuged at 5000 rpm for 30 minutes. The water-rich phase was stirred at 4°C overnight with 45 g of Amberlite IRC-718 beads in the Cu^{++} form. After centrifugation, the extract was concentrated at 30°C at reduced pressure to about 25% of the original volume and subsequently perfused through an Amberlite XAD-2 column at a flow rate of 4 ml/min. Fractions were collected every two minutes,

and samples were assayed by HPLC for 2-MBQ and 2,6-DMBQ directly and after hydrolysis and oxidation. The quinone-yielding fractions were pooled together for separation by displacement chromatography by using an octadecyl-silica column with distilled water as the carrier and 40 mM aqueous n-butanol as the displacer. The flow rate and the temperature were 0.1 ml/min and 21°C, respectively. Fractions were collected every minute and 1 µl aliquots were diluted 1000-fold with distilled water and analyzed by HPLC. The column used in displacement chromatography was regenerated by washing with 200 ml of methanol, followed by reequilibration with water.

Enzymatic synthesis of methoxyhydroquinone glucosides. 5 g of defatted wheat germ were placed in three test tubes. 12.5 ml of water was added to the first test tube. 12.5 ml of an aqueous solution containing 2 mM glutathione (GSH), 2 mM 2,6-DMHQ and 2 mM uridine diphosphoglucose (UDPG) were added to the second. The third contained 2 mM GSH, 2 mM UDPG, and 2 mM 2-MHQ. The test tubes were shaken and allowed to stand for approximately 24 hours at room temperature. The samples were analyzed by HPLC before and after incubation.

RESULTS AND DISCUSSION

In the first part of the study the presence of 2-MBQ and 2,6-DMBQ in wheat germ was investigated. Reversed phase chromatography has been previously shown to be suitable for the analysis of methoxy substituted benzoquinones (14, 15), as long as certain precautions are taken. With the amperometric detector, the minimum detection limit of both 2-MBQ or 2,6-DMBQ was about 250 femtomoles at a signal-to-noise ratio of three.

Direct HPLC analysis of aqueous wheat germ extracts showed neither 2-MBQ nor 2,6-DMBQ, nor the corresponding hydroquinones to be present. Since it is known that quinones react readily with nucleophiles such as thiols and amines, the reaction of 2-MBQ and 2,6-DMBQ with glutathione present in wheat germ at appreciable levels was also investigated. In aqueous solution 2-MBQ reacted almost instantaneously with glutathione whereas 2,6-DMBQ reacted at a much slower rate to form the corresponding hydroquinone thioether that was oxidized to the benzoquinone thioether as determined by HPLC analysis. Direct HPLC of the wheat germ extract failed to show any of the peaks corresponding to products formed in the reaction between methoxybenzoquinone and glutathione. The results suggest that such a reaction would not account for the disappearance of the quinones if they had originally been present in wheat germ.

An assay procedure for the quinones was developed based on the hydrolysis of their glucosides. The glucosides were hydrolyzed by lowering the pH of the extract to 2.5, and the hydroquinones thus obtained were oxidized by hydrogen peroxide to the quinones which were analyzed by HPLC. This procedure showed 1.0 µmoles of 2,6-DMBQ per gram of dry wheat germ but no 2-MBQ. However, upon addition of trace amounts of cupric nitrate to the extract 3.5 µmoles of 2-MBQ per gram of dry wheat germ were found by HPLC analysis. Figure 1 shows chromatograms of the extracts that were obtained with and without cupric nitrate. Thus, catalytic amounts of copper ions were required for the decomposition of the relatively stable 2-MHQ conjugate, and the effect is likely due to complexation of 2-MHQ with copper (16, 17).

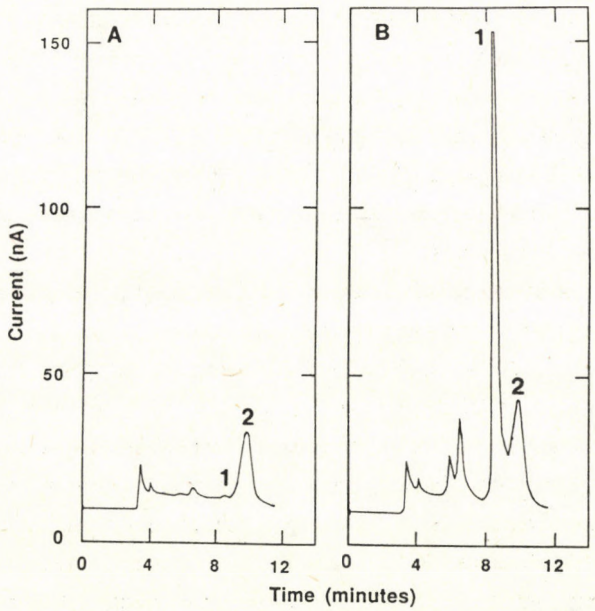

Fig. 1. Chromatograms of wheat germ extract treated with 0.3% hydrogen peroxide at pH 2.5 (A) without and (B) with 0.5 mM $Cu(NO_3)_2$ added. Column: 250x4.6 mm 5 µm octadecylsilica. Mobile phase: 50 mM NaH_2PO_4, pH 3.0, 15% (v/v) acetonitrile.
Flow rate: 1.0 ml/min. Detector potential: -200 mV vs. Ag/AgCl. 1 = 2-MBQ, 2 = 2,6-DMBQ

With a method for the assay of 2-MBQ, 2,6-DMBQ and their conjugates in hand, a variety of vegetables were also analyzed for these substances. However, they were found to be present only at the parts per billion level and wheat germ contained by far the highest concentration of such substances.

Hydrolytic and oxidative treatment of the fractions obtained by fractionation of wheat germ extract by HPLC and subsequent HPLC analysis of the samples showed that 2-MBQ was formed from three substances and another three yielded 2,6-DMBQ. In view of earlier studies the six compounds were assumed to be glucosides of the corresponding methoxyhydroquinones.

Extraction with water, rather than with acetonitrile, methanol, acetone and chloroform, gave the highest yield of these products. Unfortunately, wheat germ is very hygroscopic and one gram of wheat germ readily absorbs over 1 ml of water, which cannot be removed by pressing or centrifugation. Furthermore, the conjugates were not stable in the aqueous extract, probably due to the presence of β-glucosidase. Upon incubation at room temperature for 24 hours, all of the quinone-yielding substances disappeared with the concomitant liberation of 2,6-DMHQ in the extract. In order to minimize losses, extraction was carried out at $4^{\circ}C$.

Upon addition of acetonitrile to the aqueous extract, a large fraction of protein precipitated and two liquid phases were formed due to the limited miscibility of acetonitrile with the aqueous solution having high sugar concentration. Over 95% of the quinone-yielding products were found in the water-rich phase but its residual protein content caused excessive foaming during the concentration step. The proteins were removed by treatment with the metal chelation resin, Amberlite IRC-718 in copper form. Unlike Cu^{++} in free solution, the chelated copper did not catalyze the decomposition of the conjugates to the corresponding quinones. Therefore by this treatment, the preparation of a concentrated solution suitable for chromatographic separation was facilitated.

After removing 75% of the solvent by rotary evaporation, the extract was passed through the Amberlite XAD-2 column. The macroreticular, non-polar polymer removed the less polar con-

stituents from the extract and the quinone-yielding substances, presumably glucosides, eluted together with a large amount of carbohydrates. It is noted that wheat germ consists of 45% carbohydrates, more than one third of which are comprised of sucrose and raffinose (18, 19) and they were only partly removed by treatment of the extract by acetonitrile.

The isolation of quinone yielding glucosides using a separation technique which has selectivity for the sugar residue would be difficult under such conditions. Whereas polyphenolic glycosides have been separated by chromatography on dextran gels (20), the high sugar content of the wheat germ extract also precluded the use of this technique. Therefore, reversed-phase chromatography was chosen and in order to process sufficient quantities without the use of preparative columns, displacement chromatography was employed (21). In this mode of chromatography, after introduction of the sample to be separated, the column is perfused by a solution of a displacer, which has a greater affinity for the stationary phase than any of the feed components. In this non-linear chromatographic process, the components separate into adjacent bands, eventually forming a displacement train (22). With the octadecyl-silica column several displacers such as 2-(2-n-butoxyethoxy)ethanol, tetrabutylammonium bromide, and n-butanol were tested and n-butanol was found to give the best results. Figure 2 shows that three major components were obtained with greater than 99% purity as measured by HPLC analysis of the fractions. In comparison with the analysis of the feed, the yields of components 1, 2 and 3 were 1.57 mg, 3.52 mg and 6.47 mg representing recoveries of 97%, 84% and 33%, respectively.

Identification of the three major products was made by FAB-mass spectrometry and by ^{1}H-NMR. Molecular weights of the three compounds were found to be 302, 464, 626, respectively. The ^{1}H-NMR spectra confirmed their identity as methoxyhydroquinone glucosides. The NMR spectra of the two compounds with molecular weights of 302 and 626 were examined, and they both contained the following peaks: a doublet at 6.80 ppm (one aromatic H), a doublet at 6.77 ppm (one aromatic H), a doublet at 6.63 ppm (one aromatic H), and a singlet at 3.80 ppm (three

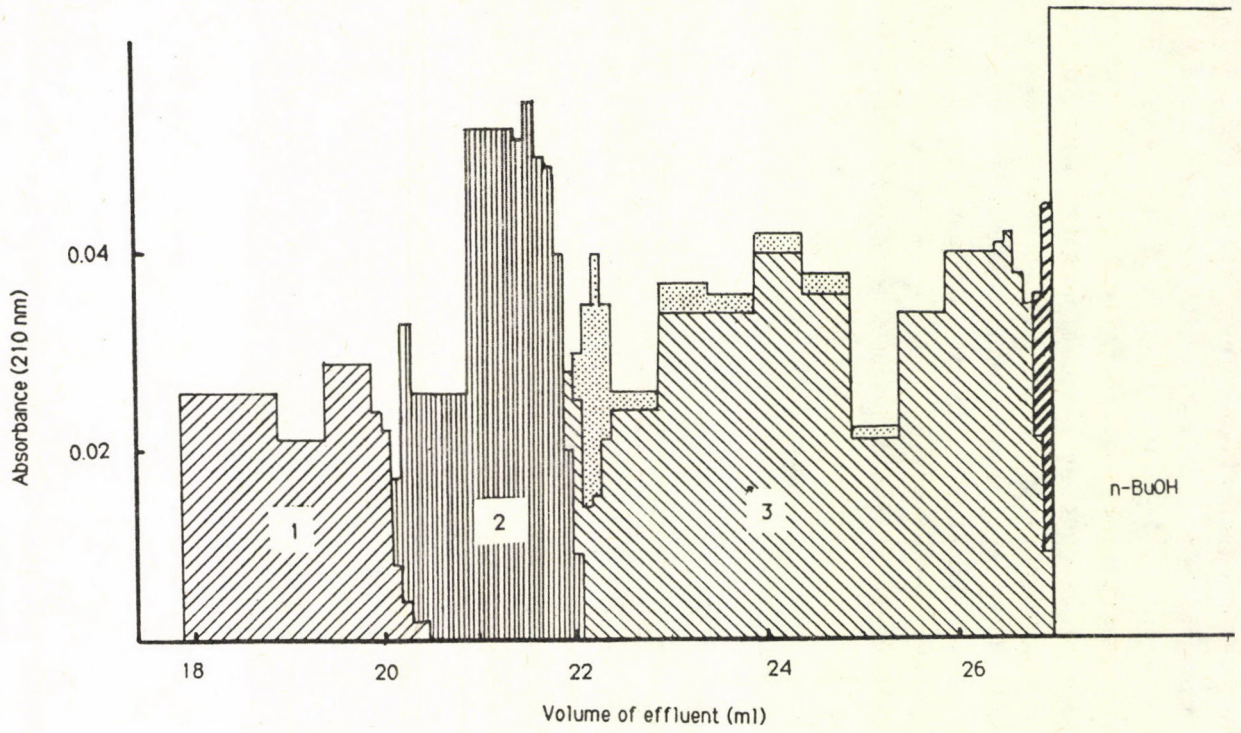

Fig. 2. Displacement chromatogram of glucosides of 2-MHQ present in wheat germ extract. Carrier: water. Displacer: 40 mM n-butanol. Column, 5 µm octadecyl-silica, 250x4.6 mm, flow rate, 0.1 ml/min. Bands of the major components: 1, (3-methoxy-4-hydroxyphenyl)-β-glucopyranoside, 2, (3-methoxy-4-hydroxyphenyl)-β-cellobioside, and 3, (3-methoxy-4-hydroxyphenyl)-β-cellotrioside

H's,-OCH_3). The doublets on the MW = 626 spectrum at 4.34 and 4.40 ppm are characteristic of β-(1 → 4) bonds between two glucose molecules, and the doublet at 4.97 ppm is due to the hydrogen next to the aromatic ring. Thus both mass spectra and NMR spectra confirmed the identity of the isolated compounds as (3-methoxy-4-hydroxyphenyl)-β-glucopyranoside, (3-methoxy-4-hydroxyphenyl)-β-cellobioside and (3-methoxy-4-hydroxyphenyl)-β-cellotrioside. The mass spectrum and NMR spectrum of the cellotrioside are shown in Figures 3 and 4.

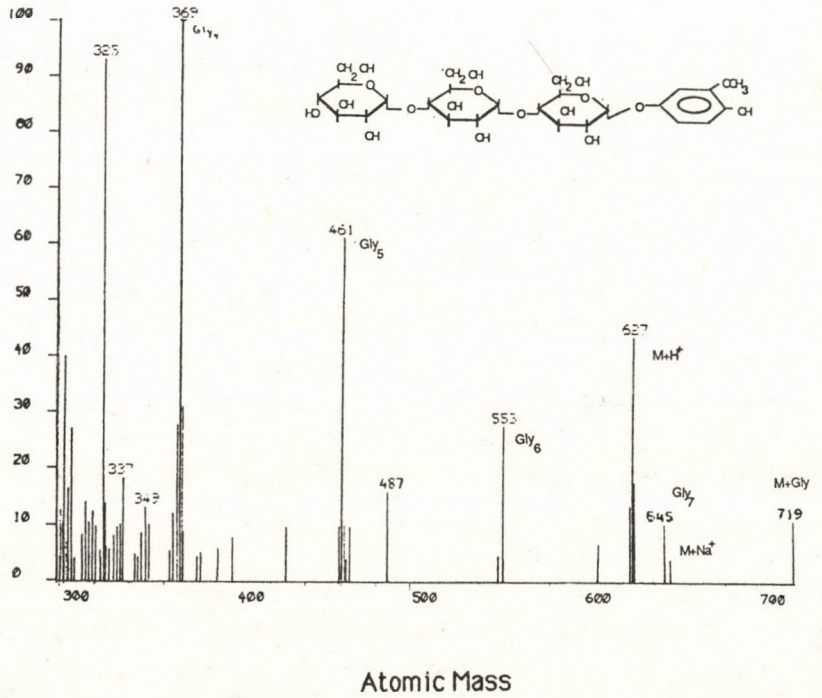

Fig. 3. FAB mass spectrum of (3-methoxy-4-hydroxyphenyl)-β-cellotrioside. Gly = glycerol

Of the three glucosides obtained by the displacement chromatography, only the cellotrioside was found to be present in the fresh wheat germ extract. After an hour at room temperature, most of the cellotrioside was hydrolyzed to glucopyranoside and cellobioside probably by β-glucosidase. It was found that addition of the copper chelated resin to the extract arrested the decomposition of the glycosides.

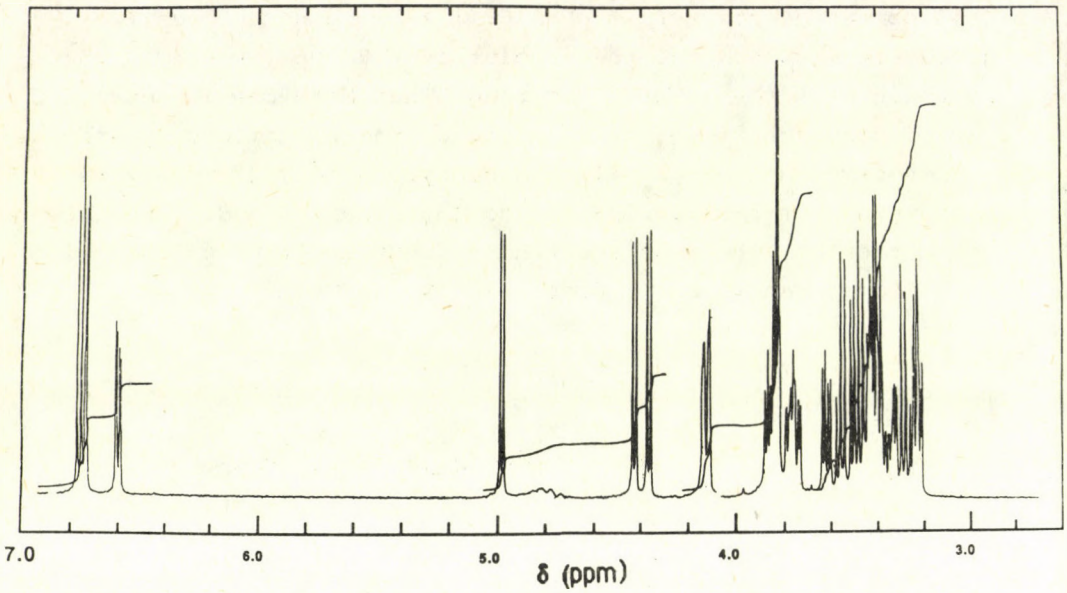

Fig. 4. ^{1}H-NMR of (3-methoxy-4-hydroxyphenyl)-β-cellotrioside is deuterium oxide

HPLC analysis of fresh wheat germ extract suggested that glucosides of 2,6-DMHQ were also present but they were found to decompose much more readily than the glucosides of 2-MHQ. Adventitious metals as well as the stainless steel wetted surface in the chromatographic system accelerated the decomposition. The isolation of the 2,6-DMHQ conjugates, therefore, would required another procedure including the immediate removal of glucosidase activity from the extract and the use of a metal free system for chromatographic separation.

In order to investigate the enzymatic synthesis of the MHQ glucosides in the wheat germ extract, it was incubated with 2-MHQ and uridine diphosphoglucose. Glutathione was added to avoid oxidation of the hydroquinone. The results are illustrated by the chromatography shown in Figure 5. It is seen that the peaks representing MHQ-glucopyranoside and MHQ-cellobioside increased presumably due to their enzymatic synthesis. By incubating the extract with 2,6-DMHQ under the same conditions, an increase of two peaks corresponding to DMHQ-pyranoside and DMHQ-cellobioside were also noted.

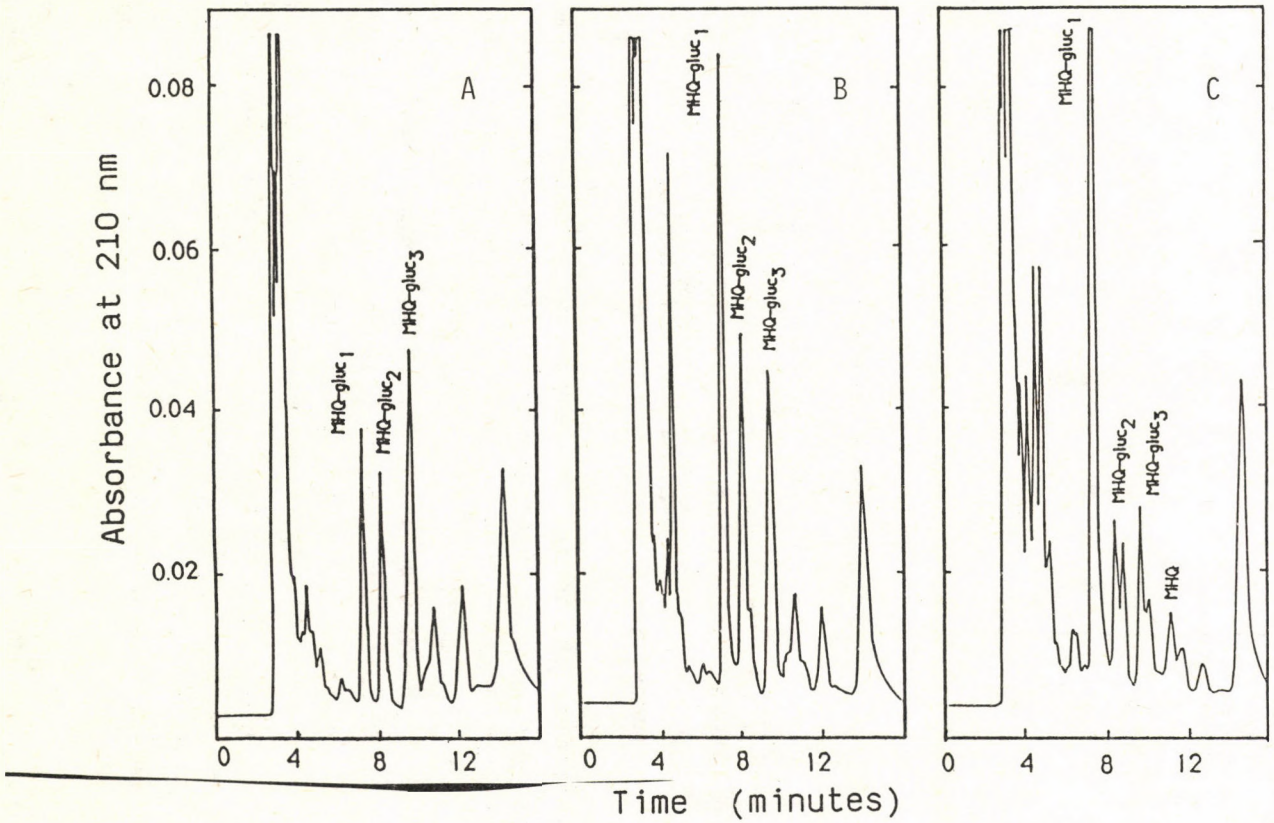

Fig. 5. Chromatograms of (A) wheat germ extract incubated at room temperature after one hour, (B) wheat germ extract with 2 mM 2-MHQ and 2 mM uridine diphosphoglucose added after one hour incubation at room temperature, and (C) wheat germ extract with 2 mM 2-MHQ and 2 mM uridine diphosphoglucose after incubation at room temperature for 24 hours. Mobile phase: 50 mM NaH_2PO_4, pH 6.0, 4% (v/v) acetonitrile added. Column: 5 µm octadecylsilica, 250x4.6 mm

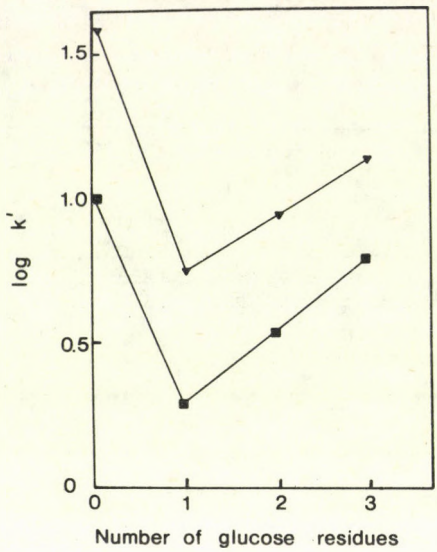

Fig. 6. Plot of the logarithmic retention factors against the number of glucose residues in 2-MHQ and 2,6-DMHQ glucosides. Column: 5 µm octadecylsilica, 250x4.6 mmµ mobile phase, 50 mM NaH_2PO_4, pH 6.0, 4% (v/v) acetonitrile, flow rate: 1.0 ml/min, temp. 25°C. ▼ DMHQ, ■ MHQ and the respective glucosides

The retention behaviour of the methoxyhydroquinone glucosides in reversed phase chromatography is illustrated in Figure 6. For the glucosides, the logarithmic retention factor increases linearly with the number of sugar residues whereas, the retention of the aglycon (n = 0) is higher than that of the conjugates. This can be explained by a large increase in polarity upon addition of the first glucose to the hydroquinone which results in a lower retention. On the other hand, addition of subsequent sugar moieties drastically changes the molecular size without significantly increasing the polarity and the retention of the oligoglucosides increases with the number of glucose residues in the molecule.

Acknowledgements

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TWO ALTERNATE FORMS OF CARDIAC MYOSIN AS CONTROLLED BY THYROID HORMONE IN THE RAT *

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A study has been made of some properties of the myosin heavy chain as controlled by thyroid hormone in the rat. Thyroid saturation leads to the exclusive presence of the α-form, athyroidy to β-myosin. Their relevant ATPase rates differ by 2.93:1.

Two-dimensional electrophoresis of the cyanogen bromide peptides in the weakly alkaline range of isoelectric focussing displays 5 pairs of difference peptides between the α and β-form. These are allocated to the helical tail of the myosin molecule.

INTRODUCTION

Effects of thyroid hormone, TH, upon the myocardium were observed by Beznak (1) and Korecky (2), who described contractility changes in the rat heart occurring upon hypophysectomy. Biochemical analysis was introduced by Rovetto et al (3) who observed that in hypex rats the molar myosin-ATPase rate, assayed at moderate ionic strength and with Ca^{++}, is reduced by some 40 percent; the original ATPase was restored, or beyond, by TH but not by growth hormone. Application of the method of gel electrophoresis of native myosin (d'Albis, 4; Hoh, 5) explained this by the demonstration of a set of 3 isomyosins V_1, V_2, and V_3, of decreasing electrophoretic mobility. The interpretation (d'Albis, 6; Hoh et al, 7) is that, with identical MLC's, these isozymes represent the homo- or hetero-dimeric combinations of two types of MHC's, αα, αβ and ββ respectively. The athyroid state leads to ββ, euthyroidy to

* *In memoriam Albert Szent-Györgyi*

variable proportions of α and β as dependent on the species and developmental stage; and saturation with TH to αα. The ATPase under the stated conditions is higher for α than for β, about 3-fold.

The occurrence of the different isozymes is ascribed to the selective expression of one or the other of two members of the myosin (MHC) gene family, and the α, β manifestation is due to the preferential expression of the corresponding genes, as set forth by Nadal-Ginard (8) and Mahdavi et al (9,10). In this paper, we describe a characterization of the α-β distinction in terms of pairs of difference peptides as detected by two-dimensional mapping of MHC split by CNBr at the methionine sites.

METHODS

Preparation of Myosin

Our standard laboratory procedures (e.g., Mommaerts et al, 11) were employed in the preparation of rat ventricular myosin.

Ca^{++}-Activated ATPase Activity

The method used was our standard laboratory procedure as described by Buller et al (12). ATPase activity was measured at 25° C in a medium containing 100 mM KCl, 1mM $CaCl_2$, 1 mM ATP, and 50 mM Tris-acetate, pH 8.0. The reaction was stopped by addition of 0.25 volume of ice-cold 10% trichloracetic acid. Precipitated protein was removed by centrifugation, and inorganic phosphate in the supernate determined.

Electrophoresis of Isomyosins at Low Ionic Strength

Given the solubility characteristics of myosin, this requires solubilization, as achieved by d'Albis (4) and Hoh (5). Originally, ATP was used as the solubilizing agent; later it was replaced by pyrophosphate. The method gives good separation of the cardiac isomyosins, both as detected by protein staining or by visualized ATPase reaction. Fig. 2A gives an example. We used the specific details of d'Albis et al. (6). Myosin samples (7 μg) were run on cylindrical gels (6 x 76 mm) of 4% acrylamide and 0.1% bisacrylamide. The running buffer was composed of 0.04 M tetrasodium pyrophosphate, 1 mM EDTA, 10% glycerol, 0.01% 2-mercaptoethanol, pH 8.5. Electrophoresis was performed at a constant voltage of 14 volts cm^{-1} for 22 hours at 1.5-

2.0°C.

The gels, after staining with Coomassie Blue and destaining, were scanned densitometrically, and quantitated planimetrically.

CNBr Peptide Mapping Procedures

The CNBr digestion of myosin was performed as described in our previous paper on myosin heavy chain peptide mapping (Mommaerts et al, 11, p. 135-137). For the first-dimensional isoelectric focusing separation, we have altered the gel solution, so as to produce a more nearly linear pH gradient in the basic region. The modified gel solution was obtained as follows: the pH 8-10.5 ampholine was increased from 0.2 to 0.6 ml and the pH 4-6.5 ampholine, from 0.4 to 0.6 ml in 30 ml of gel solution. The pH 3-10 ampholine remained the same (1.4 ml in 30 ml).

Subfragments of Myosin

The distribution of difference peptides among various fragments of T^+ myosin, LMM, HMM, and S1 was studied on myosin from rat ventricles, whereas T^- myosin (athyroid state) was so studied on ventricles from dog heart. We chose the latter because large amounts of tissue are available, and the peptide maps of dog myosin in the euthyroid state and rat T^- myosin (athyroid state) are essentially identical in respect to the difference peptides in question.

The preparation of dog cardiac myosin was that of Flamig and Cusanovich (13, 14), starting with the heart in relaxing solution (0.025 M KCl, 0.04 M Na tetraborate, 0.005 M EDTA, pH 7.0) immediately after excision from the bled animal.

Preparation of Myosin Subfragments

Dog cardiac S1 was prepared with the method of Siemankowski & White (15), and the same was used on rat myosin.

Experiments on Rats

These were done on Sprague Dawley rats obtained from Hormone Assay in Chicago. The age was 8-10 weeks.

RESULTS

Changes in Myosin Components and ATPase during Transitions in Thyroid Status

Athyroidy (T^-) and eu- or hyperthyroidy (T^+, i.e. TH saturation) were established as described. The former was attained under the conditions of thyroidectomy or hypophysectomy and the latter by the administration of thyroid hormone, specifically T_4, to either form of T^- animals. In the athyroid state, the myosin-ATPase declined toward an end value of about 0.30 μmols. $min^{-1}.mg^{-1}$ over a period of several weeks. Twenty-one days after surgery, T_4 was then administered (2.0 μg per 100 g body weight), and the ATPase changed toward a terminal value of about 0.88. The end value usually exceeded that for the euthyroid state, inasmuch as in the latter, increasingly with age, a mixed isomyosin composition prevails (Lompre et al, 16).

Electrophoretic determination of isomyosins showed the following. After elimination of thyroid function, whether in thyrex or hypex experiments, there was a conversion from a predominantly V_1 to a V_3 composition, while the opposite conversion occurred upon administration of TH. In both directions, there was a transient appearance of V_2, which is a lesser component going through a plateau or maximum of about 0.20 of total, in either direction. The results obtained are in keeping with the view of Rovetto et al (3) that TH is the principal determinant, but bring out that the euthyroid state in the adult rat does not saturate the response system.

Presentation of Peptide Maps

Overall CNBr peptide maps show that differences occur between the α and β forms, obtained respectively from T_4-supplemented normal rats (all α) and hypex or thyrex rats (all β); and also for normal dog ventricular myosin. The latter, which electrophoretically is all β, shows some difference spots due to species characteristics, but will be found similar to rat β in certain comparisons to be made below.

Leaving the description of the complete patterns to our previous paper (16), we restrict ourselves to peptides in the weakly alkaline region of the maps, around the isoelectric focus region pH 6-8 and with molecular weights of about 15 kdalton and less. There is excellent and

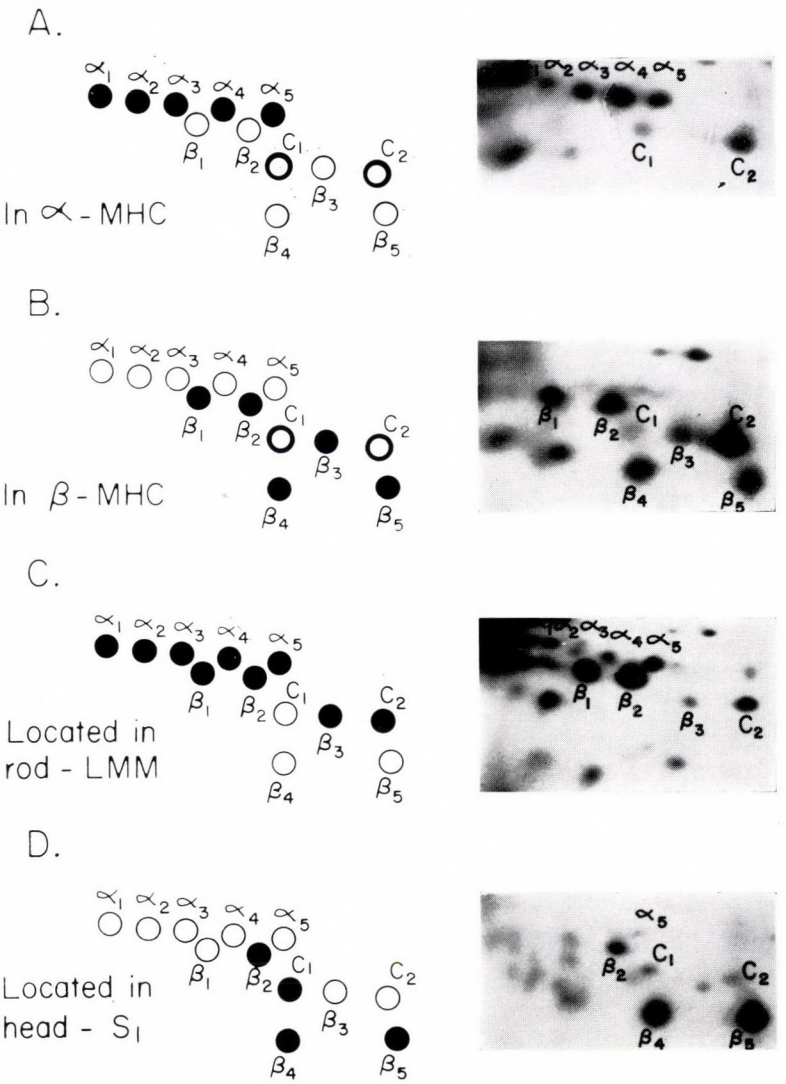

Fig. 1. Left hand figures: diagramatic identifications; right hand figures: actual photographs of gels. From top to bottom: A: α-myosin MHC; B: β-myosin MHC; C: myosin helical rod portion, LMM; D: myosin head portion, S_1

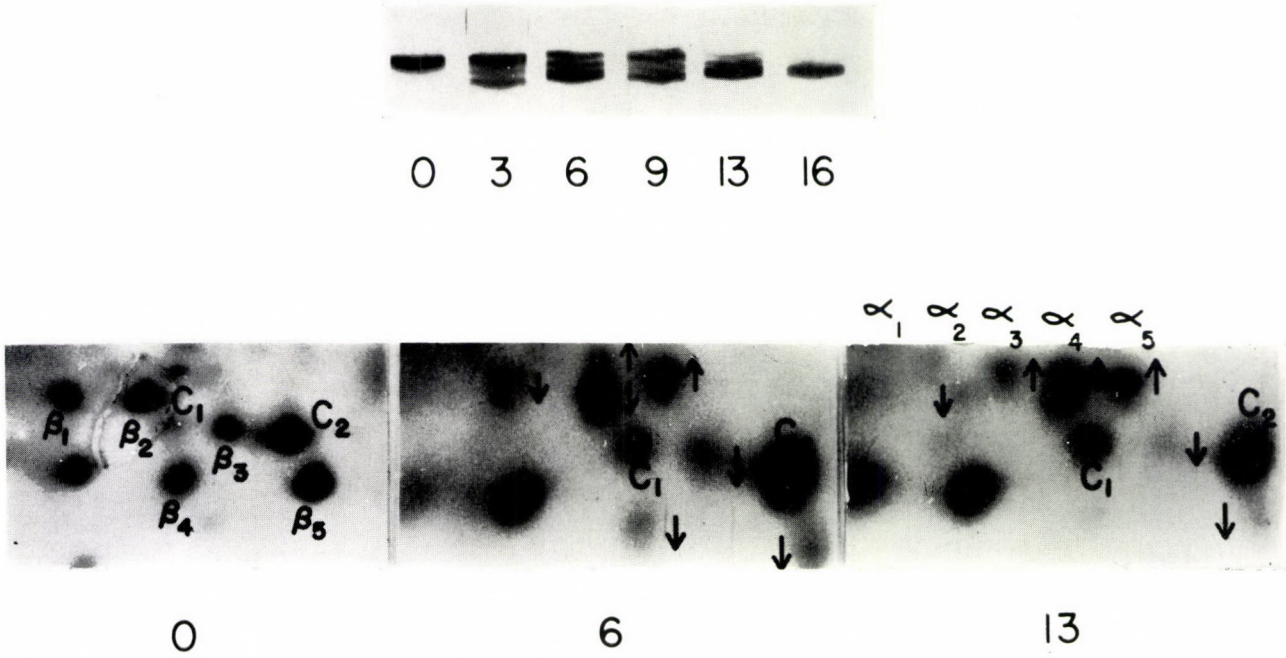

Fig. 2. Changes occuring during transition from athyroid to hyperthyroid condition. Top figures: Albis-Hoh patterns at times in days after start of TH administration; Bottom figures: peptide maps at three stages of the same transformation

reproducible resolution of some of the major spots, two of which (C_1 and C_2) are constant and not affected by the thyroid status. As to others, they reflect distinct peptide replacements involved in the α-β transition: upon attainment of the athyroid state, five peptides α1-α5 (Fig. 1A) are found to be replaced by five others, β1-β5 (Fig. 1B).

Transitional stages are illustrated in Fig. 2. This includes d'Albis-Hoh diagrams (Fig. 2A) at different points in the β to α transformation upon administration of T_4, illustrating the primary data Fig. 2B shows peptide maps for three moments in the transition. The gradual replacement of the α 1-5 by the β 1-5 peptides is clearly seen. These difference peptides are assigned to different domains in the MHC-molecule, by separate analysis of the LMM fraction (including also whole rod-sections) and of subfragment-1 as described in the Methods section. The alkaline peptides of the α-protein are in S1 (not illustrated). The difference peptides α1-α5 are all recovered in the helical rod fraction (Fig. 1C), but of their β-counterparts β 1 and 3 are present only in the rod (Fig. 1C); the others are in S1, or distributed over both fractions (Fig. 1D). This seeming paradox is examined in the Discussion. The sum of the masses of the α1-α 5 peptides plus C_2 is of the order of 110 kdalton, and thus accounts largely for the helical rod domain (110 kdalton), while being in excess with respect to LMM only (single chain 70 kdalton); thus they extend into S2.

DISCUSSION

Isomyosins and ATPase

Approximate ratios of α- and β-ATPase rates have been indicated in the literature, e.g. about 3 for rat cardiac myosin (Pope et al 17), 2.6 for separated subfragment S1 of rabbit (Ueda and Yazaki, 18), and 2.5 for bovine myosins (Flamig & Cusanovich, 13, 14).

On the basis of the experimental findings, we have analyzed the relation between ATPase and α, β isozyme composition in mixtures of the electrophoretic components V_1, V_2, and V_3, as appearing the the TH-controlled transients. The ATPase rate (R) can be expressed as:

$$R = k_1[V_1] + k_2[V_2] + k_3[V_3]$$

where k_1, k_2, and k_3 are molar rate coefficients of the myosin ATPase, and $[V_1]$, $[V_2]$, $[V_3]$ the concentrations. Recalling that V_1 and V_3 are

.e homodimers of α and β respectively, and that V_2 is the heterodimer, one would predict that the coefficient for $[V_2]$ would be an average of k_1 and k_3 if there is no allosteric interference between the α and β chains. A standard regression analysis, by means of the SAS statistical analysis software package, was used to estimate the three coefficients. The best fit for the ATPase rate equation was:

$$R = 0.88[V_1] + 0.78[V_2] + 0.30[V_3]$$

This gives the value of 2.93 for the ratio k_1/k_3. However, due to the small proportion of $[V_2]$ in the mixtures, the value of k_2 is not accurate enough to discuss the consequences of its deviation from $(k_1 + k_3)/2$, which would be indicative of allosteric interaction between the α and β chains in the heterodimer.

Structural Interpretations

While we have developed our CNBr-peptide mapping procedures over the last ten years, there has been, beginning in Gergely's laboratory (Sreter et al, 19), a parallel evolution of peptide patterns obtained by enzymatic digestion. While originally we selected our approach because it was not dependent upon conformational effects, including those due to interaction with MLC's, we are now favorably impressed by further developments in the enzymatic methodology, both when used 2-dimensionally (Whalen et al, 20) or 1-dimensionally (Chizzonite et al, 21; Srihari et al, 22). However, our method has proven itself well with respect to the detection of difference peptides encountered between the thyroid-controlled α and β-forms of MHC in the selected region. We shall limit our discussions to the peptides ascribed to the helical-rod part of the molecule, because there is less available knowledge concerning the head part (S1). The peptides in question are all placed in the neutral or weakly alkaline range of the isoelectric gradient separation of the peptides, and have been indexed as α 1-5 and β 1-5, plus two constant peptides C 1-2 present in both states (Figs. 1A and 1B).

We must first call to attention that, while for this set of peptide pairs the α-peptides are all in the LMM moiety of the MHC, most of the β-peptides, nos. 2, 4, and 5 are partly or wholly found in the S1-fraction. This would suggest that they, and their α analogs, are

located in the S1 - S2 transition region, an area for which there is now some information, for cardiac MHC, due to the cDNA or genomic nucleotide sequences obtained by Kavinsky et al (23). This work demonstrates a specific methionine in α at their amino acid positions 1320 and possibly 2212, and in β at position 1156, and there may be others not yet identified because the respective cloned-DNA's overlap only partly. The explanation of our peptide allocation is evidently that proteases split the α and β myosins differently. This may be due to different chymotrypsin sensitive groups, e.g. a tyrosine at nucleotide position 1195 in MHC-β but it is just in this region that the available sequences of α and β cease so that we cannot examine this further; or it may be due to different degrees of hindrance exerted by MLC's. These differences in the presumed site of proteolytic splitting were obtained from the rat and dog, respectively.

We make a specific proposal regarding the hot-spot of peptide differences found at 1/6 peptide chain length from the carboxyl terminal. Mahdavi et al (9, 10) record that the putative β-form has an additional methionine at amino acid position 1659 (Nadal-Ginard's notation) which would replace a peptide of about 12 kdalton by one of 10.8 kdalton. The structures in question are:

```
                1650
MetGluIleGlnLeuSerGlnAlsAsnArgIleAlaSerGluAlaGlnLysHisLeuLysAsn
                                                  1670        1754
                                                  AlaGlnAlaHisLeuLys..Met

MetGluIleGlnLeuSerHisAlaAsnArgMetAlaAlaGluAlaGlnLysGlnValLysSer
                                                  LeuGlnSerLeuLeuLys..Met
```

while the C-terminal is at position 1961 and 1959 for α and β, respectively (Nadal-Ginard et al, 7, 9, 10).

This difference pair would be represented by peptide spot α-5 versus β-3 or β-4 and it must be β-3 because this is the one difference peptide which is not relegated to the S1-S2 transition region. Their relative positions are best seen from fig. 2B, central panel, where α-5 and β-3 occur side by side.

From the known sequences (Mahdavi et al, 9, 10), the molecular weights are 12,090 (α-5) and 10,910 (β-3). From the vertical positions

of the peptide spots, they are 16.2 kd and 12.5 kd respectively. These correspondences are well within the range of variations commonly encountered in electrophoretic molecular weight determinations in urea without sodium dodecyl sulfate.

Peptide α-5 is more acidically placed on the map than β-3. Under the proposal, it differs from β-3 by having a Glu at position 1650, and His at 1666 and 1673. At its location in the gradient (pH about 7.2), the His residues are likely to be mostly uncharged, but the Glu is negative. We remark, however, that it is impossible to precisely assign pK-values and IEP's without extensive structural information. See Matthew et al (24); Friend et al (25); and the extensive review by Matthew et al (26).

It is likely that our difference peptides, allocated to the LMM-region, are contained among the difference peptides described by Flink et al (27), but their two-dimensional technique differs from ours and no direct comparison is possible.

Transitions Between Thyroid States

We refer to the experimental section and single out these noteworthy points.

Both in the α to β transition following the establishment of athyroidy, and in the reverse change caused by administration of thyroid hormone, there is a gradual replacement of one MHC by another, judged equally in terms of d'Albis-Hoh band analysis, and in terms of ATPase activity. The fractional amount of myosin that is in the V_2 form, i.e. the α, β-heterodimer, is always relatively small. This could result from a lesser affinity in the heterodimeric combination, or from the occurrence of mostly alpha or beta in different cells, for which there is elegant immunohistochemical demonstration (Gorza et al, 28, 29; Schiaffino et al. 30). However, there are cardiocytes that contain both myosins simultaneously (Weisberg, et al, 31), and the heterodimer thus occurs in those. The half-life of myosin in this system is 9 days (Rabinowitz, 32); thus, the kinetics of mutual replacment of the isomyosins is quite slow, and comparable to the turnover times. The reprogramming of isogene selection, as expressed in messenger replacement, is much faster, after a lag period of a few hours (Everett

et al, 33), for the rabbit.

ENDOCRINOLOGICAL QUESTIONS

While there are no indications of differences between hypex and thyrex in the time course of the iso-MHC transformations as indicated by ATPase measurements, there are such suggestions in the transformations detected by d'Albis-Hoh electrophoresis. This issue is complex, because after thyroidectomy there would still be a presence of pituitary factors which, directly or indirectly (e.g. via somatomedins; see Lakshmanan et al, 34), would modify the responses to added hormone. None of the goals set for this present investigation, however, are affected by these limitations.

We note that in the rat, euthyroidy corresponds to a preponderance of α-MHC but with distinct amounts of β (Hoh, et al, 7; Schwartz et al, 35) which increase with age. Postnatally, however, this emerges from a perinatal all-β composition. It is assumed but not proven that fetal and adult β are identical, and we have reasons to question this. In other animals, sometimes after transient perinatal changes, the adult MHC is β (Lompre, et al, 17). In the human, we find β throughout, from the earliest accessible fetal stage onward (10 weeks of gestation, Mommaerts unpublished). There is, however, a distinct change, postnatally, in the β-actomyosin ATPase rate, which may (Schier and Adelstein, 36) or may not be a function of an MLC difference. We have preliminary evidence that one of the β-specific peptides is absent in fetal-β, and that the fetal β-form can be recalled in the adult by physiological regulations. Euthyroid dog cardiac myosin, with respect to the diagnostic α, β difference-peptides considered here, is indistinguishable from rat β-myosin (Fig. 1C), and becomes α in hyperthyroidism (Flink et al, 27). The latter two points reinforce the conclusion that in the larger mammals, the normal euthyroid myosin is indeed, structurally, a true β-myosin.

It is noteworthy that the normal euthyroid state can have such vastly different connotations with respect to the saturation or unsaturation of receptors in individual target organs, and in different species.

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ABBREVIATIONS

We speak of thyroid hormone, TH, when the distinction between the effects of T_3 or T_4 has not been made or is not crucial in the context. Other abbreviations: Hypex, hypophysectomy; thyrex, thyroidectomy; MHC, MLC, myosin heavy and light chain; S1, S2, HMM and LMM, myosin subfragment-1(head), -2 (hinge), heavy and light myosin, respectively; CNBr, cyanogen bromide.

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ASCORBIC ACID AND LIPID PEROXIDATION: THE CROSS-OVER EFFECT*

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Results are presented concerning the effects of ascorbic acid on models of lipid peroxidation in rat liver microsomal suspensions. The models used are (a) NADPH-dependent; (b) NADPH-CCl_4-dependent; (c) cumene-hydroperoxide-dependent. Lipid peroxidation was measured using the thiobarbituric acid reaction. Ascorbic acid affects these lipid peroxidation systems in a non-linear manner. Evidence is provided for a pro-oxidant effect of ascorbic acid at low concentrations, and an antioxidant effect at high concentration. Possible mechanisms for this cross-over effect are discussed.

INTRODUCTION

Vitamin C was isolated (as hexuronic acid) by Albert Szent-Györgyi in 1927 (1) whilst working in Cambridge with Gowland-Hopkins, and identified in 1933 (2). It is a water-soluble acidic substance (pK_a 4.18; see ref.3) that has a number of key functions in metabolism (4) and in the body's defensive armoury (5). In this paper, dedicated with affection and gratitude to Albert Szent-Györgyi, we consider some aspects of the anti-oxidant properties of ascorbic acid and point out where, on occasion, this substance may act in a pro-oxidant manner.

Vitamin C can function in defensive reactions in our cells and tissues through its action as an antioxidant, thereby inhibiting a variety of free

*In memoriam Albert Szent-Györgyi

radical mediated reactions. A characteristic consequence of the production of reactive free radicals in biological systems is the *initiation* of free radical chains that may result in serious cell damage, even cell death (6). An example is the process known as lipid peroxidation in which polyunsaturated fatty acids (PUFAH's) are successively degraded to a variety of biologically reactive products (7) :

$$R\bullet + PUFAH \longrightarrow PUFA\bullet + RH \quad [1]$$

$$PUFA\bullet + O_2 \longrightarrow PUFAOO\bullet \quad [2]$$

$$PUFAOO\bullet + PUFAH \longrightarrow PUFAOOH + PUFA\bullet \quad [3]$$

$$PUFAOO\bullet, PUFAOOH, \text{metal ions} \longrightarrow \text{products} \quad [4]$$

$$PUFA\bullet, PUFAOO\bullet \longrightarrow \text{non-radical products and less reactive radical products} \quad [5]$$

Reaction [1] is initiation by the reactive radical R•; reactions [2-4] are chain propagation, and reactions [5] are chain terminations. Antioxidants have been classified (8) conventionally as preventive antioxidants that basically interfere with the supply of the reactive initiating radical R•; and chain-breaking antioxidants that interfere with reactions [2-4] by reactions that fall within the overall category of reaction [5]. Ascorbic acid is a water-soluble chain-breaking antioxidant.

Another property of ascorbic acid has to be considered in relation to radical reactions in biological material. Ascorbic acid is a strong reducing agent and can convert Fe^{3+} to Fe^{2+}. Since ferrous ions catalyse the production from H_2O_2 of the very reactive and highly damaging hydroxyl radical by the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO\bullet \quad [6]$$

it is clear that ascorbic acid can allow Fe^{3+} to re-cycle to Fe^{2+} and so ensure the production of more HO• radicals. In this type of specific sense, ascorbic acid can act as a pro-oxidant. The change-over of a substance's action as an antioxidant to a pro-oxidant (or vice-versa) has been called

the cross-over effect (9). In this paper we provide some experimental results on the cross-over action of ascorbic acid in rat liver microsomes.

METHODS

Adult male Wistar rats of weight 250g ± 50g supplied by Charles River, Margate, Kent, U.K. were used. They had been fed on a RM3 expanded diet supplied by the Special Diet Services, Witham, Essex.

Liver Microsome Preparation

The rats were starved overnight but were given water ad libitum and were killed the following morning by stunning followed by cervical dislocation. The livers were removed quickly, dried, weighed and cooled in an ice-cold solution of 0.25M sucrose. They were homogenised using a Potter-Elvehjem homogeniser (5 strokes) in a volume of 0.25M sucrose equivalent to 5-times the weight of liver. After centrifuging the homogenate at $11,700g_{av}$ at 4°C for 10 minutes the supernatant was recentrifuged under the same conditions. The supernatant from this second spin was carefully removed and was centrifuged again at $168,000g_{av}$ for 40 minutes at 4°C under vacuum. The microsomal pellet obtained was rinsed free of overlying supernatant with ice-cold 0.1M tris-HCl buffer of pH 7.4 and was then kept in the dark using aluminium foil wrapping and stored at -70°C until required.

Stimulation Systems for Lipid Peroxidation

Lipid peroxidation of rat liver microsomes was stimulated using two systems: NADPH/carbon tetrachloride (10) and cumene hydroperoxide (11) stimulation.

i) Carbon tetrachloride stimulation: A standard stock solution was prepared to generate NADPH and contained the following components: 84.9mM KCl; 37.7mM tris-HCl buffer of pH 7.4; 5.7mM glucose phosphate (monosodium salt); 0.24mM $NADP^+$ (disodium salt); glucose 6-phosphate dehydrogenase (0.25U/ml) and 106mM acetamide in a total volume of 95.4ml. Standard stock (2.2ml) was added to 0.5ml of L-ascorbic acid in stoppered glass tubes; solutions of L-ascorbic acid were adjusted to pH 7.4 using 0.1M NaOH.

Microsomes were resuspended in ice-cold 0.15M KCl such that 1ml of suspension contained microsomes equivalent to 1g wet weight of original rat liver and 0.3ml of this suspension was added to the mixture. Lipid peroxidation was stimulated with a final concentration of 6.9mM carbon tetrachloride; 10µl of a CCl_4:DMSO (1:4) mixture were added using a Hamilton micro-syringe, and 8µl DMSO was added to the controls. Tubes stimulated with NADPH/CCl_4 were performed in triplicate while those stimulated endogenously were performed in duplicate. All tubes were incubated for 15 minutes in a shaking water bath in the dark at 37°C. The reaction was stopped by adding 6ml of ice-cold 10% trichloroacetic acid (TCA).

ii) Cumene hydroperoxide stimulation: Different concentrations of L-ascorbate (pH 7.4) up to a final concentration of 20mM were prepared and 0.15ml was added to a mixture (1.1ml) of 0.15M KCl and 0.1M tris-HCl buffer at pH 7.4 in the ratio of 2:1 respectively. A solution of cumene hydroperoxide (70% in cumol) was diluted 4,500 times with double distilled water such that the final concentration was 100µM when 0.15ml was added to the reaction tubes. Controls contained 0.15ml buffer instead of cumene hydroperoxide. A microsome suspension was prepared as above and after an addition of 0.1ml the tubes were incubated in an identical manner previously described except the reaction was stopped by precipitating the protein with

only 3ml of ice-cold 10% TCA.

Determination of malondialdehyde

Lipid peroxidation was evaluated (12) by the production of material giving a positive reaction with thiobarbituric acid (TBA). Precipitated protein was removed by bench centrifugation at $1,000g_{av}$ for 10 minutes and 2ml of the clear supernatant was heated with 2ml of 0.67% TBA in a boiling water bath for 10 minutes. After cooling the solutions the absorbance at 535nm was determined. Results were expressed as nmoles MDA produced/mg protein/minute incubation by using a value of 1.49×10^5 ($1.mol^{-1}\ cm^{-1}$) for the molar extinction coefficient of the MDA-TBA adduct. The amount of protein present in the microsomal supernatant was determined using the method of Lowry et al (13) using bovine serum albumin as standard.

Materials

The chemicals purchased from BDH Chemicals Ltd., Dagenham, Essex, U.K. were potassium chloride;trichloroacetic acid;thiobarbituric acid; sucrose; L-ascorbic acid and carbon tetrachloride. Chemicals obtained from Sigma Chemical Company, Poole, Dorset, U.K. were acetamide; D-glucose 6-phosphate (monosodium salt); trizma base; dimethyl sulphoxide, DMSO and bovine serum albumin fraction V.

The cumene hydroperoxide was purchased from Fluka, Glossop, Derbyshire, U.K. β-$NADP^+$ (disodium salt) and glucose 6-phosphate dehydrogenase yeast grade (II) suspension were obtained from Boehringer Mannheim, Lewes, Sussex, U.K.

RESULTS

The system involving carbon tetrachloride-stimulated lipid peroxidation (Figure 1) with a range of ascorbate concentrations up to 10mM demonstrates a cross-over phenomenon whereby a pro-oxidant effect is observed at low concentrations and an antioxidant effect as the concentration is increased. This has been found to be true in both the endogenous stimulation, involving NADPH, and in the stimulation tubes with NADPH/CCl_4; in both cases the cross-over point is between 1mM and 2mM ascorbate. Maximal stimulation of MDA production occurs at 200μM and is about 60% higher than the controls.

With the cumene hydroperoxide-stimulated system (Figure 2) the maximal stimulation (after an initial decrease) for both endogenous (containing no cumene hydroperoxide) and stimulated tubes occurs at about 1mM. It is apparent by comparing the endogenous values of both the cumene hydroperoxide and carbon tetrachloride systems in Figures 1 and 2 that at concentrations of ascorbate higher than 5mM there is an inhibition of MDA production in

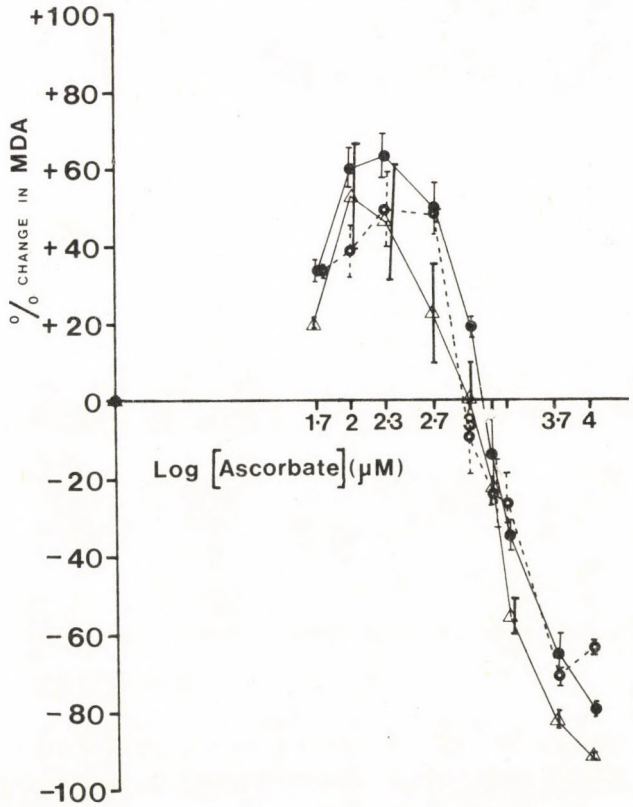

Fig. 1. The effect of ascorbate on (i) O---O NADPH (endogenous) stimulated (ii) ●——● NADPH/CCl_4 stimulated and (iii) Δ——Δ NADPH/CCl_4 minus endogenous (resultant stimulation) stimulated malondialdehyde production in rat liver microsomes. The microsomes had been incubated at 37°C for 15 mins in the presence of ascorbate and NADPH generating stock, and either with or without carbon tetrachloride induced stimulation of lipid peroxidation. Ascorbate concentration is represented as the $\log_{10}$ of that concentration in μM. The percentage change in MDA production was calculated relative to nmol MDA produced/mg protein/minute incubation in controls which did not contain any ascorbate. Each point shows the mean of the percentages of six individual experiments ±S.E.M. rather than the percentage of the average malondialdehyde production.

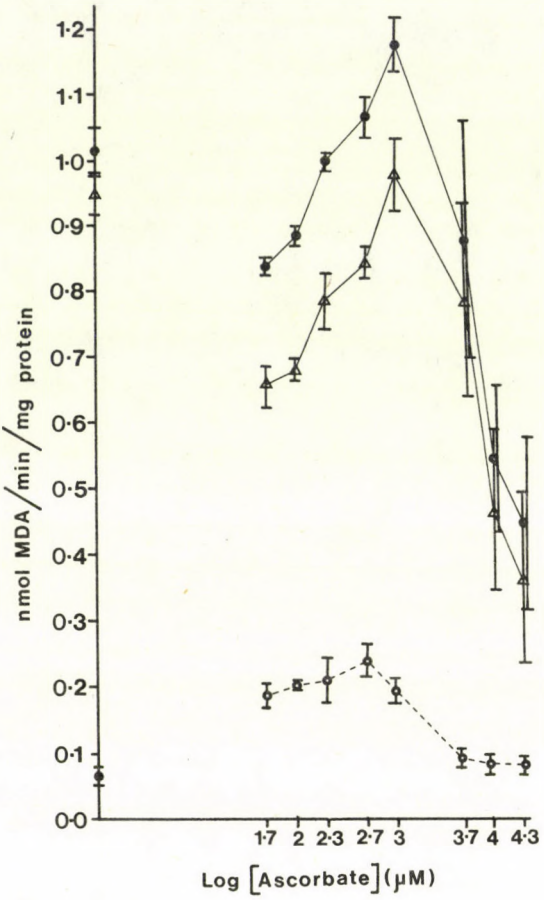

Fig. 2. The effect of ascorbate on malondialdehyde production stimulated (i) ●——● with cumene hydroperoxide (stimulated) and (ii) O---O without cumene hydroperoxide (endogenous) in rat liver microsomes. (iii) Δ——Δ shows the resultant stimulation. The microsomes had been incubated at 37°C for 15 minutes in the presence of ascorbate, cumene hydroperoxide and buffer. The extent of lipid peroxidation was evaluated as nmol MDA produced/mg protein/min incubation. Ascorbate concentration is represented as the $\log_{10}$ of that concentration in μM, with the exception of the origin which represents an ascorbate concentration of 0 μM. Each point represents the mean of 3 experiments ±S.E.M.

the presence of NADPH (Figure 1,--●--) but not in the absence of NADPH (Figure 2,--●--).

The results given in Figure 2 illustrate that cumene hydroperoxide-stimulated lipid peroxidation does not show a decisive pro-oxidant effect with ascorbic acid over the range tested. There is some evidence that very low concentrations of ascorbate inhibit the cumene hydroperoxide system (Figure 2; range 0-50μM ascorbate) followed by an apparent pro-oxidant phase; at ascorbate concentrations greater than 5mM only an antioxidant effect is evident.

DISCUSSION

The antioxidant activity of ascorbic acid shown in Figures 1 and 2, for concentrations of about 3mM or greater, is consistent with many previous reports demonstrating that vitamin C is an inhibitor of degradative free radical reactions. Vitamin C has long been known to 'protect' food against autoxidation (14), and although it is a hydrophilic chain-breaking anti-oxidant (5) it can serve to regenerate the lipophilic α-tocopherol (15) : the antioxidant activity of ascorbic acid is a result of the relative stability (16) of the semi-dehydroascorbate radical (SDA•) formed in the scavenging reaction that removes the reactive radical R• :

$$R\bullet + \text{Ascorbate} \longrightarrow RH + SDA\bullet \qquad [7]$$

The results reported here for the effect of low concentrations of ascorbic acid on two different lipid peroxidising systems demonstrate a non-linear relationship between ascorbate concentration and the extent of peroxidation. At low concentrations of ascorbic acid there is evidence (Figure 1) for a pro-oxidant effect of ascorbic acid that may result from the reducing properties of this antioxidant. Ascorbate can reduce Fe^{3+} to Fe^{2+} as mentioned in the Introduction. The presence of Fe^{2+} can result in the production of the strongly oxidising radical, hydroxyl (HO•) from H_2O_2, and in the formation of reactive lipid peroxyl (PUFAOO•) and lipid

alkoxyl-(PUFAO•) radicals from lipid hydroperoxides (PUFAOOH) :

$$Fe^{3+} + AA \longrightarrow Fe^{2+} + SDA\bullet + H^{+} \quad [8]$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + HO\bullet \quad [9]$$

$$Fe^{2+} + PUFAOOH \longrightarrow PUFAOO\bullet, PUFAO\bullet \quad [10]$$

It is worth noting that iron-catalysed breakdown of lipid hydroperoxides preferentially yields alkoxyl radicals with Fe^{2+}, and peroxyl radicals with Fe^{3+}. The presence of ascorbate would thus favour the chemically more reactive, and more powerful initiating alkoxyl species due to reduction of Fe^{3+} to Fe^{2+}. Moreover, ferrous ions in the presence of a number of biologically occurring substances such as thiols can autoxidise readily to yield the superoxide anion radical

$$Fe^{2+}(SH) + O_2 \longrightarrow Fe^{3+} + O_2\bullet^{-} \quad [11]$$

and this can undergo dismutation to H_2O_2

$$2O_2\bullet^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad [12]$$

followed by reaction [9]. The above discussion has concentrated on iron-catalysed reactions, but other metal ions can play similar roles. Thus, if the parent system under study contains traces of iron, and some endogenous peroxide and oxygen, then in the presence of a low concentration of ascorbate reactions [8-12] can proceed to result in increased lipid peroxidation; higher concentrations of ascorbate introduce an antioxidant effect through reactions [13] :

$$PUFAOO\bullet, PUFAO\bullet + AA \longrightarrow PUFAOOH, PUFAOH, + SDA\bullet \quad [13]$$

Although these results demonstrate a cross-over effect for ascorbate in the two systems studied *in vitro*, it is important not to transfer these findings uncritically to situations *in vivo*. In the whole animal situation there is a family of antioxidant protective mechanisms including α-tocopherol, bilirubin, ubiquinol, β-carotene, ascorbate, urate and protein thiols. This family appears to function as a 'buffer-complex' that protects the

body against oxidative stress. The resultant reactions, whilst being individually intrinsically simple, form a delicately tuned and powerful defensive combination: Albert Szent-Györgyi would have loved to unravel the complexities and subtleties involved.

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DETERMINATION OF QUINONE–REDUCTASE ACTIVITIES IN WHOLE CELLS AND PURIFIED ENZYMES FROM FREE RADICAL DECAY KINETICS*

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Under physiological conditions, ascorbate is able to reduce quinones with a similar midpoint potential only very slowly. Concentrations of ascorbyl and semiquinone free radicals as high as several micromoles·(dm)$^{-3}$ with half–lives in excess of 10^3 seconds can result from such slow reactions. Previous studies have shown that viable cells can eliminate the free–radical populations produced when ascorbate and 2,6–dimethoxy–p–benzoquinone are combined, and that cells of a transformed (cancerous) phenotype are much more effective in this elimination process than comparable cells of normal phenotype. It is shown here by analysis of the reaction kinetics that the concentration of free radicals observed in the ascorbate–quinone mixtures is a trace of the residual quinone. The rate of elimination of the free radicals by viable cells is therefore a measure of their total quinone reductase (diaphorase) activity, reflecting the sum of contributions from both one– and two–electron quinone reductases.

INTRODUCTION

The role of electron–accepting agents in the control of cell division formed the last subject of study in Albert Szent–Gyorgyi's laboratory (1,2). Of primary interest was the role that naturally occurring quinones and ascorbic acid played in this process (3). Szent–Gyorgyi theorized that cancer resulted from a breakdown in the balance between oxidative and reductive processes in the cell (4), a balance that he believed might be restored in transformed cells by supplementation of appropriate oxidative cofactors. A constituent of wheat germ, 2,6–dimethoxy–p–benzoquinone (DMQ), was believed to be such a cofactor.

In memoriam Albert Szent-Györgyi

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Previous studies showed that a fresh solution of DMQ and ascorbate exhibited an antitumor activity in Ehrlich ascites tumor–bearing mice (5). Electron spin resonance (ESR) experiments showed that this antitumor activity was correlated with the production in the reaction mixture of ascorbyl and semiquinone free radicals, which reached a peak concentration of approximately 4 micromole·$(dm)^{-3}$ and which had a half–life exceeding 10^3 seconds.

In order to understand more clearly the nature of the interaction between viable cells and the free radicals, washed cells were exposed to ascorbate–DMQ mixtures *in vitro* (6) and the fate of the free radicals was studied using ESR. It was found that viable cells speeded up the disappearance of radicals from the reaction mixture through NAD(P)H– and SH–dependent enzymic processes (7). Furthermore, experiments on five cultured cell lines showed that cells of transformed (cancerous) phenotype were much more potent in speeding free radical decay than were directly comparable cells of normal phenotype (8). Preliminary experiments on cellular fractions derived by differential centrifugation, subsequent protein separation studies, and observations using commercially obtained oxidoreductases led to the conclusion that this phenomenon arose from unidentified differences in oxidoreductase activities between normal and transformed cells (9,10).

In order to exploit for research or diagnostic purposes the finding that transformed cells can eliminate free radical populations more rapidly than their normal counterparts, it is necessary to understand not only the mechanism of formation of free radicals in ascorbate–DMQ mixtures but also the ways in which cells can accelerate their decay. Progress has been made in understanding the generation and decay of the radicals in cell– and enzyme–free solutions (11). This article extends the analysis to allow conclusions to be drawn from the changes in free–radical decay kinetics brought about by cells and enzymes.

MATERIALS AND METHODS

The technique for the generation of the ascorbyl and DMQ–semiquinone free radicals was the same as reported previously (9). Briefly, 1 ml of 100 mM HEPES buffer solution (pH 7.4) containing 5 mM ascorbate, 1 mM EGTA, 1 mg superoxide dismutase (Sigma) and 0.5 mM DMQ was mixed from concentrated stock solutions. The DMQ was added last, at time $t = 0$, to activate the free radical generation process. In studies of concentration dependencies, the levels of ascorbate and DMQ were varied from these nominal values.

After activation with DMQ, the reaction mixture was immediately syringed through a fine tube into the bottom of a solution cell positioned in the microwave cavity of an ESR spectrometer (Varian E–109). The field–frequency lock of the spectrometer had been previously tuned to the down–field line of the resolved doublet of the ascorbyl radical or to an inverted line of the semiquinone radical 3.44 G upfield from this ascorbate feature; the spectrometer frequency was 9.52 GHz. The intensity of the chosen spectral feature was recorded as a

function of time from $t = 30$ seconds until $t = 1400$ seconds. A modulation of 0.5 G was found to be optimum for these studies on the separately resolved free radicals. Although the semiquinone feature was cleanly separated from the ascorbyl resonance spectrum, the ascorbyl feature slightly overlapped one of the semiquinone resonance lines and therefore contained a small intensity contribution from it; this small overlap was taken into account when the time dependency of the ascorbyl radical concentration was analyzed.

RESULTS AND DISCUSSION

The primary reaction that occurs on mixing ascorbate (AA) and DMQ is the reduction of DMQ to its corresponding hydroquinone (DMHQ) and the accompanying oxidation of ascorbate to dehydroascorbate (DHA):

$$AA + DMQ \rightarrow DHA + DMHQ .$$

The final equilibrium concentrations of the components can be expressed in terms of kinetic parameters k_1 and k_2 such that

$$k_1 \cdot [AA]_{eq} \cdot [DMQ]_{eq} = k_2 \cdot [DHA]_{eq} \cdot [DMHQ]_{eq} .$$

If the starting concentrations of DMQ and AA are Q_0 and A_0, respectively, and the proportion of DMQ that has been reduced to DMHQ at time t is $r(t)$, then it can be shown (11) that the reaction follows the kinetic equation

$$r(t) = 2c \cdot \{b + (b^2 - 4ac)^{1/2} \cdot \coth(k \cdot t)\}^{-1} ,$$

where

$$a = 1 - k_2/k_1$$
$$b = 1 + A_0/Q_0$$
$$c = b - 1$$

and where the overall reaction rate constant, k, is a function of the rate constants k_1 and k_2 and of the initial concentrations of the reagents:

$$k = k_1 \cdot Q_0^2 \cdot \{ 1 + 2(A_0/Q_0) \cdot (2k_2/k_1 - 1) + (A_0/Q_0)^2 \}^{1/2} .$$

If $k_1 \sim k_2$ and $A_0 >> Q_0$, as was the case for the ascorbate and DMQ studies

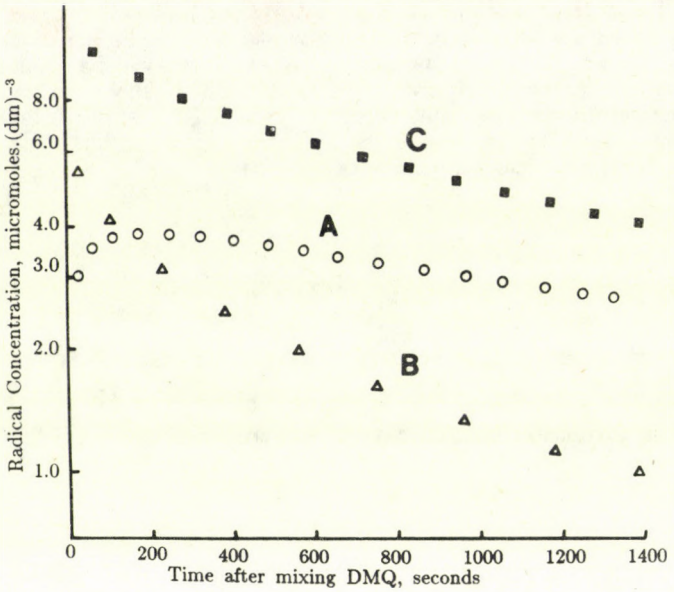

Figure 1. Time dependence of the concentration of semiquinone (A) and ascorbyl (B) free radicals in a solution initially containing 5 mM ascorbate and 0.5 mM DMQ. A weighted combination of these signals (C) was used in earlier cell and enzyme studies (see text).

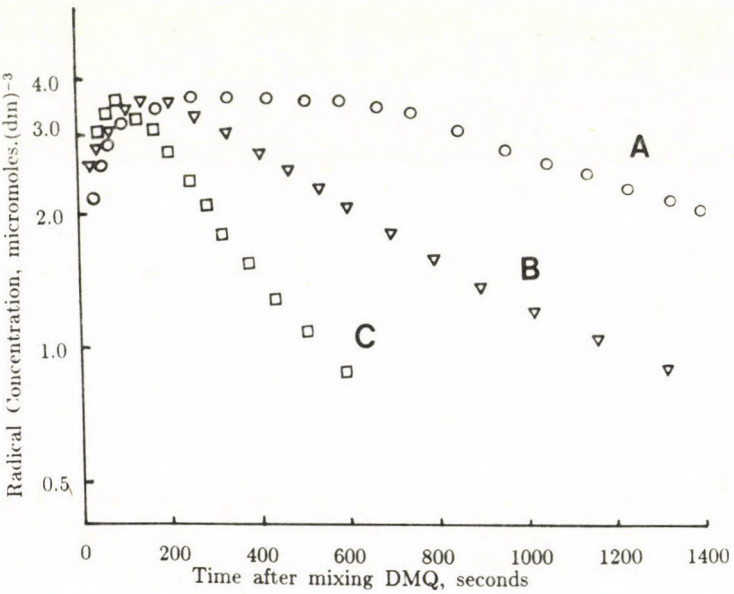

Figure 2. Time dependence of the concentration of semiquinone free radicals in DMQ–ascorbate solutions as a function of the initial ascorbate concentration. (A) A_0 = 5 mM, (B) A_0 = 10 mM, (C) A_0 = 20 mM.

reported both here and earlier, then this approximates to

$$k \sim k_1 \cdot Q_0 \cdot A_0 ,$$

predicting that the kinetics should be first order in both the ascorbate and quinone concentrations.

It has also been shown (11) that the semiquinone free radicals $Q^{\cdot}$ observed during the course of the reaction result from the disproportionation process

$$DMQ + DMHQ = 2Q^{\cdot}$$

which leads to a concentration of semiquinone, $q^{\cdot}(t)$, given by

$$q^{\cdot}(t) = (k_3 \cdot [DMQ] \cdot [DMHQ])^{1/2}$$

where [DMQ] and [DMHQ] are the instantaneous concentrations of the quinone and hydroquinone at time t and [DMQ] + [DMHQ] = Q_0 at all times. Figure 1 illustrates the time dependency of the concentration of the ascorbyl and semiquinone free radicals in a reaction mixture where A_0 = 5 mM and Q_0 = 0.5 mM. As the reaction proceeded, and DMQ was converted to DMHQ, the concentration of $q^{\cdot}(t)$ at first rose, peaked (when the concentrations of DMQ and DMHQ were equal), then fell toward a final equilibrium level. Figure 2 shows the semiquinone time dependence $q^{\cdot}(t)$ for different initial ascorbate concentrations A_0. As predicted by the equation, the peak level of semiquinone in the reaction depended solely on the initial concentration of quinone, Q_0, and was independent of the starting concentration of ascorbate, A_0. As expected from the discussion of the constant k given earlier, however, the rate with which the reaction proceeded depended on the ascorbate level. By monitoring the residual quinone in the reaction mixture using UV/visible light spectroscopy at 397 nm (11), the disproportionation process was shown to remain in equilibrium at all stages of the reaction between the quinone and ascorbate. Evidently this equilibrium process is much faster than the rate with which ascorbate interacts with DMQ. The parameter k_3 had the value 2.56 x 10^{-4} at pH 7.4, leading to a peak semiquinone concentration $q^{\cdot}_{max}$ of 4 x 10^{-6} M for a starting quinone concentration Q_0 = 0.5 mM.

Figure 3 illustrates the time dependency of the ascorbyl free radicals, $a^{\cdot}(t)$, in ascorbate–DMQ mixtures as a function of the starting concentration of ascorbate, A_0. The mechanism responsible for the production of the ascorbyl radicals

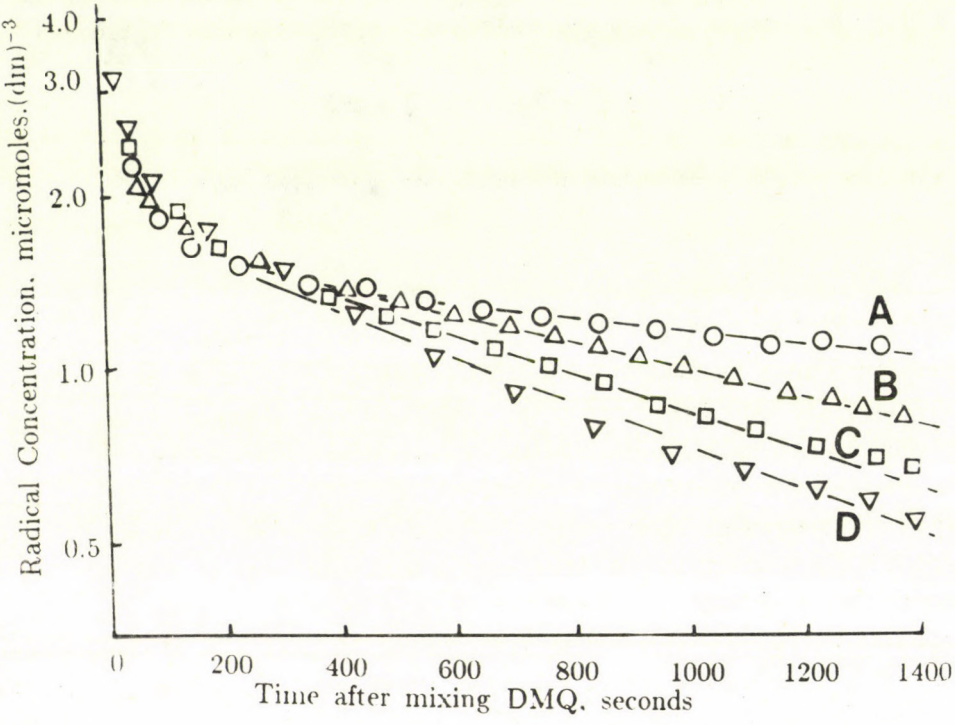

Figure 3. Time dependence of the concentration of ascorbyl free radicals in DMQ–ascorbate solutions as a function of the initial ascorbate concentration (A) A_0 = 2 mM, (B) A_0 = 3 mM, (C) A_0 = 4 mM (D) A_0 = 5 mM.

appeared to be different from that responsible for the semiquinone because the ascorbyl concentration was not related to the corresponding disproportionation reaction

$$a^{\cdot}(t) = (k_4 \cdot [AA] \cdot [DHA])^{1/2} .$$

Instead, inspection of Figure 3 suggests that the ascorbyl radical concentration reflected more closely the rate of the overall reaction, $dr(t)/dt$. When $A_0 >> Q_0$, this rate was approximately proportional to the product of A_0 and the residual quinone, so that the concentration of ascorbyl free radicals was close to

$$a^{\cdot}(t) = d \cdot A_0 \cdot [DMQ] ,$$

where d is a constant. The fact that the ascorbyl radicals did not appear to arise from a straightforward disproportionation reaction is itself a matter of interest. Experiments on mixtures of DHA and AA in the absence of quinone showed that $k_4 << 10^{-8}$, a surprisingly low value for the disproportionation constant in view of the ease with which ascorbyl free radicals can be generated under mildly oxidizing physiological conditions. Two alternative interpretations of this finding can be offered. The first is that the disproportionation constant was low because of intrinsic properties of the DHA and ascorbate molecules. In this case, the ascorbyl free radicals observed so frequently under physiological conditions must arise as the result of an unusual ability of ascorbate to act as a one–electron reducing agent and because ascorbyl free radicals are subsequently eliminated by a particularly slow dismutation reaction. An alternative interpretation is that the low value of the disproportionation constant arose from a lower–than–expected oxidative activity of DHA after it was formed in aqueous solution owing to a subsequent chemical change. Carbon–13 nuclear magnetic resonance studies showed recently that DHA in its electrochemically active quinonoid form is unstable in aqueous solution (12). An equilibrium between five forms of DHA was observed, with 99% existing as a hydrated hemiketal (12). This aqueous derivative of DHA can be expected to have a greatly diminished oxidative activity compared with its parent molecule.

Thus DHA produced in the oxidation of ascorbate by DMQ can be expected to react with the aqueous solution to form a species with a very low electrochemical activity. In this case, the presence of transient ascorbyl free radicals in the ascorbate–DMQ mixtures could be related after all to a disproportionation between ascorbate and the DHA that has not yet reacted with

water. The disappearance of the ascorbyl radicals could then be explained by the subsequent reaction of DHA with water and the associated removal of the conproportionating ascorbate–DHA couple. The occurrence of such a process could help to resolve the long–standing controversy over whether the AA + DHA system can form a well–behaved (reversible) electrochemical couple. If the latter interpretation of the production of ascorbyl radicals is correct, it follows that ascorbate can form a well–behaved couple with DHA but that such a couple will be very short–lived because of the instability of DHA.

In the studies reported earlier on viable cells and enzymes (5–9), the free radical kinetics were measured using an ESR modulation width of 4 G and with the field–frequency lock of the spectrometer set to the peak of the resulting resonance line. This modulation level provided a good signal–to–noise ratio which, in turn, gave highly reproducible data and allowed the use of a fast recorder response time. The resulting broad ESR signal, however, represented a weighted sum of the ascorbyl and semiquinone radicals and did not contain resolvable features unique to each. From the equations presented above, such a weighted sum, $n'(t)$, can be written as

$$n'(t) = e \cdot a'(t) + f \cdot q'(t) = e \cdot d \cdot A_0 \cdot [DMQ] + f \cdot (k_3 \cdot [DMQ] \cdot [DMHQ])^{1/2}$$

where e and f are appropriate weighting constants. This sum can be expressed in terms of the proportion of quinone in the oxidized form remaining in the reaction mixture, $l(t)$, giving

$$n'(t) = Q_0 \cdot \{ e \cdot d \cdot A_0 \cdot l(t) + f \cdot [k_3 \cdot (1 - l(t)) \cdot l(t)]^{1/2} \} ,$$

where

$$l(t) = 1 - r(t) .$$

This equation, giving $n'(t)$ for the condition $A_0 >> Q_0$, implies that the intensity of the weighted ESR spectrum depends solely on the proportion of oxidized quinone remaining in the reaction mixture. This implies that the cells and enzymes that speeded up the decay of $n'(t)$ must have had a reductive activity towards the quinone or semiquinone; an activity towards the ascorbate would not have resulted in any significant change in the kinetics of $n'(t)$.

Furthermore, since the reaction

$$DMQ + DMHQ = 2Q^{\cdot}$$

results in the establishment of a very rapid equilibrium between quinone, hydroquinone, and semiquinone in the reaction mixture, any reductive activity towards the semiquinone would be just as effective in accelerating the decline of $l(t)$ as a reductive activity towards the quinone itself. Therefore it is not possible on the basis of reaction kinetics alone to distinguish whether one– or two–electron reductions were responsible for accelerating the decline of $n^{\cdot}(t)$. It may be concluded that the previously published results on cells and enzymes do not necessarily indicate a biological activity solely towards free radicals, but instead represent a measure of all activities that result in the reduction of DMQ in the reaction mixture. Such reductase processes towards quinones have been termed "diaphorase" activities.

Since the free radical signal is a trace of residual quinone in the reaction mixture, it is also evident from inspection of the equations that any biological process that resulted in the removal of quinone by a mechanism other than electrochemical reduction would also result in a decline of $n^{\cdot}(t)$. In this case the resulting kinetics would be substantially different from those discussed so far because the quinone would no longer be converted to the hydroquinone, which itself participates in the production of semiquinone radicals. For example, the rapid formation of an adduct between DMQ and glutathione (9) eliminates quinone from participation in further free radical production of the kind described above. In this case, not all of the quinone fraction that disappears from $l(t)$ will reappear in the reduced fraction $r(t)$; some will be converted to a new, electrochemically inactive form that we can designate $d(t)$. Because no new quinone is created, we can write

$$r(t) = 1 - l(t) - d(t)$$

so that the free radical kinetics will follow the form

$$n^{\cdot}(t) = Q_0 \cdot \{ e \cdot d \cdot A_0 \cdot l(t) + f \cdot [k_3 \cdot (1 - l(t) - d(t)) \cdot l(t)]^{1/2} \} .$$

It is possible to distinguish between straightforward electrochemical reduction and derivatization of the quinone by following the semiquinone kinetics. Since the concentration of hydroquinone will be lower in the reaction mixture when

derivatization occurs, the peak semiquinone concentration (which depends on the presence of both quinone and hydroquinone) will be lower than in the reductive case.

It follows from the expressions for $n'(t)$ that if the predominant biological activity present is reductive (so that $d(t)$ is negligible) then the proportion of quinone in the oxidized form remaining in the reaction mixture can be computed. For the case of $n'(t)$ falling with time, rearrangement gives the solution

$$I(t) = \{ [2h \cdot N(t) + g^2] - g \cdot [g^2 + 4N(t)(h - N(t))]^{1/2} \}/2i$$

where

$$N(t) = n'(t)/n_0$$
$$g = f.Q_0.k_3^{1/2}$$
$$h = e.d.A_0.Q_0$$

and

$$i = g^2 + h^2$$

and where n_0 is the peak signal intensity. For $A_0 = 5$ mM, $Q_0 = 0.5$ mM and under the experimental conditions employed here this reduces to

$$I(t) = 0.19\{ (1 + 3.08N(t)) - [1 + 6.16N(t).(1 - N(t))] \}^{1/2} ,$$

allowing the residual quinone to be computed from the ESR signal intensity. Careful inspection of the above expressions shows that when $N(t) << 1$ (at long times after the reaction is started) then $I(t)$ becomes proportional to $N(t)^{1/2}$. As a consequence, the signal intensity of the free radicals falls off very slowly in comparison to the decay of the quinone, allowing accurate quinone concentrations to be inferred down to the level of 10^{-6} mole.(dm)$^{-3}$ under circumstances that otherwise would make such determinations very difficult. Since optical transparency is not a requirement for ESR studies, the free radical kinetic procedure lends itself especially well to studies on cell suspensions.

CONCLUSIONS

The ability of viable cells and enzymes to enhance the decay of free radicals in solutions of 2,6–dimethoxy–p–benzoquinone with ascorbate results from reactivities towards semiqinone or quinone. Reactivity towards the ascorbate compounds present in the mixtures cannot account for any significant increase in

the free radical disappearance rate, but both one– and two–electron quinone–reductase activities can. In addition to reductive conversion of DMQ or its semiquinone to the hydroquinone, any depletion that leaves the quinone electrochemically inactive, such as adduct formation, will potentiate free radical disappearance. Thus the biological effects reported for a variety of cell types and for oxido–reductase enzymes represent a combination of all quinone reductase (diaphorase) and adduct–forming activities. Previous studies have shown that the cellular activities are NAD(P)H dependent, suggesting that diaphorase activities are dominant. The differences reported in the free radical depletion kinetics between cells of normal versus transformed phenotype therefore reflect variations in quinone reductase activities. Studies utilizing purified enzymes and their substrates have shown that several reductases can reduce DMQ, and preliminary studies with cells show that changes in DT–diaphorase (EC 1.6.99.2) content alone cannot explain the differences observed between different cell lines. Future work will attempt to establish whether other transformed cell lines show a similarly enhanced ability to reduce DMQ compared with their normal counterparts, to identify which enzymes are responsible for such differences, and to determine whether such enzyme systems play an obligatory role in the transformation process.

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THE Ca^{2+} PUMP OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM*

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> "I was always led in research by my conviction that the primitive basic functions of living matter are brought about by ions, ions being the only powerful tools which life found in the sea water where it originated." (Albert Szent-Gyorgyi, 1941).

Historical background.

The discovery of ATP hydrolysis by myosin (Engelhardt and Ljubimova, 1939; Ljubimova and Engelhardt, 1939) was soon followed by the demonstration of the unique properties of myosin B (Banga and Szent-Gyorgyi, 1941) that led to the isolation and characterization of actin (Straub, 1942, 1943). The contraction of actomyosin threads induced by ATP, K, and Mg (Szent-Gyorgyi, 1941) crowned these achievements and opened the way for the remarkable development of muscle

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Dedicated to the memory of Albert Szent-Gyorgyi, M.D., Ph.D., Prix Nobel.

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biochemistry. The scope and momentum of these discoveries within a span of a few years is even more astonishing, since they took place during the turbulent years of the Second World War in virtual isolation from the world community of scientists.

As soon as the basic elements of the contractile apparatus were in place, the problem of relaxation was addressed in a brief note by Szent-Gyorgyi (1942) that anticipates some of the later developments on the role of a cytoplasmic factor in the relaxation process. The following passage is quoted from this report (Szent-Gyorgyi, 1942).

> "...threads of myosin B show a violent contraction if suspended in a solution containing KCl (0.05 M), $MgCl_2$ (0.001 M) and ATP (adenyltriphosphate 0.17%). If such a contracted thread is washed out with water and suspended in a solution containing 0.25 M KCl and 0.001 M $MgCl_2$ no appreciable change is observed. If ATP is added now to the solution the thread swells up within a few minutes to its original size; it becomes transparent and similar to the original uncontracted thread in all respects. If the liquid is replaced by the salt solution in which the contraction was obtained, the thread contracts again. The contraction is thus reversible and ATP is essential not only for the contraction but also for the relaxation. The thread can be brought to contraction and relaxation by the variation of the KCl concentration.
>
> Mg is essential for the contraction as well as for the relaxation. In absence of Mg the contraction is sluggish and there is no relaxation at all. Only a very slight swelling is obtained as revealed by the somewhat increased transparency. At higher KCl concentrations (in presence of ATP) the thread disintegrates without much swelling.
>
> The Mg can be replaced by a dialysed extract of the muscle. Whether this action is due to the traces of Mg, possibly bound by the protein, or by some other substance, cannot be stated at present."

The "sarcoplasmic factor" of the muscle extract responsible for the relaxing effect was characterized later by Marsh (1951, 1952), Bendall (1952, 1953) and by Kumagai, Ebashi and Takeda (1955) and eventually became identified with fragmented sarcoplasmic reticulum. The regulation of the contractility is accomplished by ATP-dependent transport of calcium across the sarcoplasmic reticulum membrane (Ebashi, 1960, 1961; Hasselbach and Makinose, 1961; Ebashi and Lipmann, 1962). The process is of universal significance, not only in the control of mobility, but also in the metabolic regulation of most living cells (for review, see Martonosi, 1983).

The mechanism of Ca^{2+}-transport.

The complete molecular machinery required for the ATP-dependent translocation of Ca^{2+} from the cytoplasm into the lumen of sarcoplasmic reticulum is contained in the Ca^{2+} transport ATPase (Martonosi and Beeler, 1983). The Ca^{2+}-ATPase is present in different iso-forms in the sarcoplasmic reticulum of slow or fast skeletal, cardiac and smooth muscles, with an average molecular weight of close to 110,000 (MacLennan et al., 1985; Brandl et al., 1986, 1987).

The active Ca^{2+} transport is stoichiometrically linked to the hydrolysis of ATP. The process involves the binding of $2Ca^{2+}$ to a high affinity site on the enzyme in the E_1 conformation, followed by

transient phosphorylation of a β-carboxyl group of an aspartyl residue in the active site and the entrapment of Ca^{2+} in a $\overset{Ca}{Ca}\!\!\diagdown\!\!\diagup E_1 \sim P$ intermediate. A change in enzyme conformation from the E_1 to the E_2 state ($\overset{Ca}{Ca}\!\!\diagdown\!\!\diagup E_2 \sim P$) leads to the translocation and release of Ca^{2+} in the interior of the sarcoplasmic reticulum (Fig. 1).

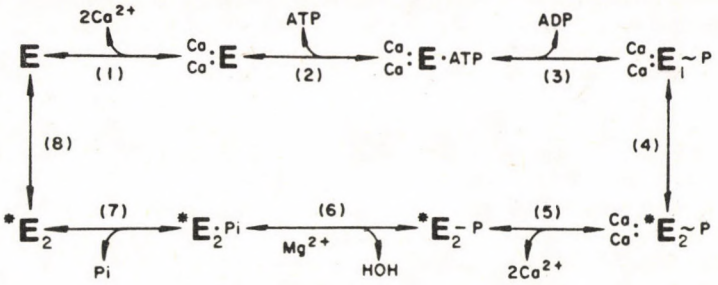

Fig. 1. Elementary steps of Ca^{2+} translocation.

The intermediates on the top line represent substructures in the E_1 state, while those on the bottom line are in the E_2 state (from de Meis and Vianna, 1979).

During this process, the affinity constant of the binding site for Ca^{2+} decreases from $\simeq 10^7\ M^{-1}$ to about $10^3\ M^{-1}$, satisfying the thermodynamic requirements for the accumulation of Ca^{2+} in the interior of sarcoplasmic reticulum. The process is completed by the Mg^{2+}-dependent hydrolysis of the E_2 - P intermediate, with the release of inorganic phosphate and the isomerization of the enzyme from the E_2 into the E_1 state (Inesi and de Meis, 1985). The Ca^{2+} transport is reversible, yielding the synthesis of 1 mole of ATP for 2 calcium ions released from the sarcoplasmic reticulum (for review, see Martonosi, 1984).

The kinetic and thermodynamic parameters of the elementary steps of the process have been determined in considerable detail, both in the forward and in the reverse reaction (Inesi, 1985), and much information is available about the regulation of the concentration and transport activity of the system under physiological conditions (Martonosi et al., 1985: 1987).

The structure of the Ca^{2+}-ATPase.

The kinetic analysis of the active Ca^{2+} transport provided a general picture of the transport process, but it is now approaching its probable limit. Further advance toward a molecular mechanism of the energy transduction and Ca^{2+} translocation requires knowledge of the structure of the pump at the molecular level.

The amino acid sequence of Ca^{2+}-ATPase isoenzymes was recently deduced from the complementary DNA sequences (MacLennan et al., 1985; Brandl et al., 1986, 1987). The structures predicted for the fast and slow isoenzymes are 84% identical. A hypothetical model of the tertiary structure of Ca^{2+}-ATPase was constructed (Brandl et al., 1986) that consists of 3 major structural regions:

1. The cytoplasmic headpiece that contains the domains for ATP binding, phosphorylation and the transduction mechanism. The latter is involved in the coupling of the energy source to the Ca^{2+}

translocation. It is assumed that the three domains are composed mainly of alternating α-helical and β-sheet structures.

2. A penta-helical stalk that is assumed to contain the high affinity binding site for Ca^{2+}. The stalk connects the headpiece to the intramembranous domain.

3. The intramembranous region that may contain as many as 10 transmembrane helices; these anchor the Ca^{2+}-ATPase in the lipid bilayer and form the transmembrane channel for the passage of Ca^{2+}.

The purpose of our studies was to define the secondary, tertiary and quaternary structure of Ca^{2+}-ATPase by electron microscope reconstruction from images of Ca^{2+}-ATPase crystals and by fluorescence, infrared, circular dichroism, and NMR analysis of the conformation and interactions of the Ca^{2+}-ATPase.

Production of two-dimensional crystals of Ca^{2+}-ATPase in the native sarcoplasmic reticulum in the E_1 and E_2 states and the reconstruction of the corresponding structure of Ca-ATPase by electron microscopy.

a. The E_2-type crystals induced by vanadate. Vanadate (V) ions, in the absence of calcium, stabilize the Ca^{2+}-ATPase in the E_2 conformation, and promote the formation of Ca^{2+}-ATPase crystals in native sarcoplasmic reticulum or in reconstituted vesicles containing the purified Ca^{2+}-ATPase (Dux and Martonosi, 1983a-e; 1984). The formation of the vanadate-induced crystals is promoted by inside-positive membrane potential, suggesting an influence of transmembrane potential on the conformation of the Ca^{2+}-ATPase (Dux and Martonosi, 1983d; Beeler et al., 1984). The crystals are disrupted by Ca^{2+} at concentrations sufficient to saturate the high affinity Ca^{2+} binding sites of the Ca^{2+}-ATPase (Dux and Martonosi, 1983c; Varga et al., 1987).

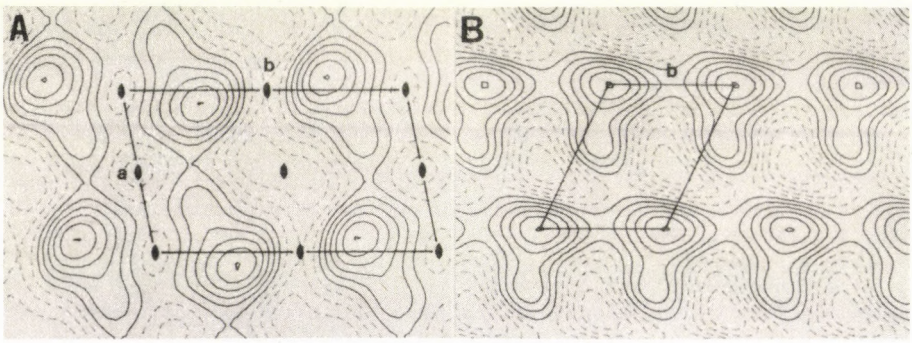

Fig. 2. Projection maps of vanadate and praseodymium-induced crystals. A. Monovanadate-induced E_2 crystals (from Taylor et al., 1984). B. Praseodymium-induced E_1 crystals (from Dux et al., 1985a). Although the two crystal forms are quite different in their oligomeric association, the Ca^{2+}-ATPase monomers have similar contours in both cases.

The E_2-type crystals contain dimer chains of ATPase molecules forming an oblique surface lattice with a space group of P2 (Taylor et al., 1984, 1986a,b; Peracchia et al., 1984); the space group and the unit cell dimensions are consistent with Ca^{2+}-ATPase dimers as structural units (Fig. 2A). The Ca^{2+}-ATPase molecules are linked

together to form a dimer by a stain-excluding bridge (Fig. 3) above the cytoplasmic surface of the bilayer (Taylor et al., 1986a,b). The gap under the bridge is likely to be accessible in the native membrane to calcium, ATP and other solutes.

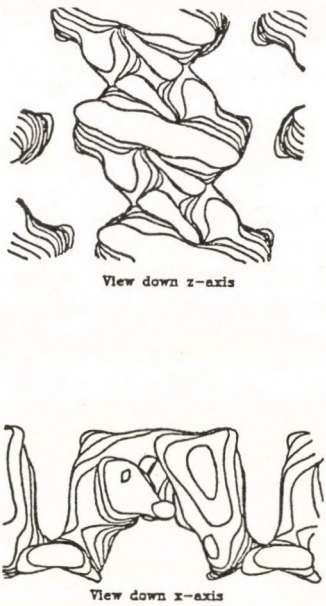

Fig. 3

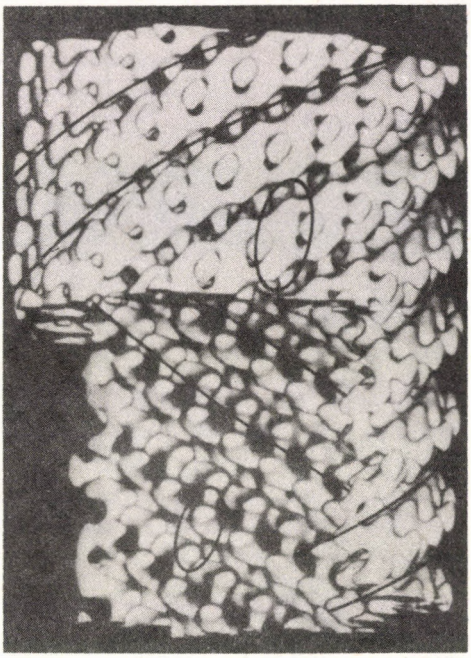

Fig. 4

Fig. 3. Surface contour views of the Ca^{2+}-ATPase from three-dimensional reconstructions of E_2-type crystals negatively stained with uranyl acetate. The drawing has been made with perspective so that features at the back appear relatively smaller than those at the front (Taylor et al., 1986a).

Fig. 4. Surface view of the vanadate-induced Ca^{2+}-ATPase crystal reconstruction from unstained, frozen-hydrated specimens. A section of the map has been cut out to reveal the inside surface of the tubule. Views of Ca^{2+}-ATPase dimers have been outlined on both the cytoplasmic and luminal surfaces. The helical path followed by a single dimer chain has been drawn in. Deep helical grooves separate Ca^{2+}-ATPase dimer chains on the cytoplasmic surface, while on the luminal surface a deep helical groove runs down the middle of the dimer chains (from Taylor et al., 1986b).

The bridge is oriented parallel with the long axis of the tubules, resulting in a uniform orientation of Ca^{2+}-ATPase dimers within the vesicles. Three-dimensional reconstruction of Ca^{2+}-ATPase molecules in unstained frozen hydrated tubules reveals both the cytoplasmic and the intramembranous regions of the protein (Fig. 4). The most striking feature of the cytoplasmic surface is the helical groove

between the dimer chains, while on the luminal surface the groove is located between the two ATPase molecules constituting a dimer. As a result, the intrabilayer domains of the Ca^{2+}-ATPase molecules within the dimer are farther apart on the luminal than on the cytoplasmic side. The reconstructions obtained from negatively stained and from unstained, frozen-hydrated specimens are essentially identical in the cytoplasmic domain.

Decavanadate binds to the Ca^{2+}-ATPase with higher affinity and stoichiometry than monovanadate, and it is more potent in inducing crystallization of Ca^{2+}-ATPase (Varga et al., 1985; Csermely et al., 1985a,b). The Ca^{2+}-ATPase crystals induced by mono- and decavanadate appear similar, but the decavanadate-induced crystals are more stable in the presence of 10^{-5} M calcium (Varga et al., 1987).

b. The E_1-type crystals of Ca^{2+}-ATPase induced by Ca^{2+} or lanthanides. A different crystal form of Ca^{2+}-ATPase develops in sarcoplasmic reticulum vesicles exposed at pH 8.0 to 10^{-5} - 10^{-4} M Ca^{2+} or 10^{-6} - 10^{-5} M lanthanides (Dux et al., 1985a). Ca^{2+} and lanthanides are presumed to bind to the Ca^{2+}-ATPase in the E_1 form. The space group of the Ca^{2+}- or lanthanide-induced crystals is P1, and the unit cell dimensions are consistent with ATPase monomers as structural units (Fig. 2). The formation of the E_1 crystals is promoted by inside-negative potential. A similar crystal form is induced by 20 mM Ca^{2+} at pH 6.0 (Dux et al., 1987).

The differences between the E_1 and E_2-type crystals indicate different types of interactions between ATPase molecules in the two conformations.

Spectroscopic studies on the conformation of Ca^{2+}-ATPase molecules in the E_1 and E_2 state.

The fluorescence of protein tryptophan (Dupont, 1976) and of the fluorescein 5'-isothiocyanate (FITC) covalently attached to the ATP binding domain of the Ca^{2+}-ATPase (Pick, 1982; Pick and Karlish, 1982) shows distinct differences, depending on the conformational state of the Ca^{2+}-ATPase. A state of high tryptophan and low FITC fluorescence is stabilized by Ca^{2+} or lanthanides, consistent with the E_1 conformation of Ca^{2+}-ATPase; EGTA + vanadate have the opposite effect, stabilizing the E_2 conformation (Jona and Martonosi, 1986). Reversible interconversion between the two conformations is accompanied by conversions between the corresponding crystal forms (Dux et al., 1985a).

The effect of transmembrane potential on the fluorescence responses of the two probes is more complex, suggesting either that more than two conformations are sensed by one or both probes, or that the response of the probes to ions or transmembrane potential contains significant local contribution that acts independently of the overall conformation of the protein (Jona and Martonosi, 1986).

Circular dichroism studies (Csermely et al., 1987) on the sarcoplasmic reticulum vesicles did not detect significant differences between the E_1 and E_2 states in the secondary structure, indicating that the $E_1 \rightarrow E_2$ transition does not involve a major net rearrangement of the polypeptide backbone of the Ca^{2+}-ATPase. The proportion of the secondary structures was estimated as follows: 45% α-helix, 7% β-sheet, 13% turn and 35% random coil. Based on these observations the $E_1 \Leftrightarrow E_2$ transition is expected to occur by local rearrangement of domains through hinge-type or relative sliding motions without refolding of the polypeptide chain (Csermely et al., 1987).

Fourier transform infrared spectroscopy offers a powerful alternative approach for the analysis of the differences between vibrational modes related to the secondary structures or side chain groups in the E_1 or E_2 conformations (Arrondo et al., 1987). By this technique a new α-helical substructure was detected, associated with the $E_1 \rightarrow E_2$ transition that appeared together with a small change in β-turns, while the β-sheet content remained essentially unaltered. There were also differences between the E_1 and E_2 states in the C=O stretching vibrations of the ester carbonyl groups of phospholipids that are not observed under identical conditions in isolated sarcoplasmic reticulum lipid dispersions. These observations imply an effect of proteins on the structure of the interfacial regions of the phospholipids, that is dependent on the conformation of the Ca^{2+}-ATPase. The two states and the corresponding spectra can be interconverted reversibly by changing the Ca^{2+} concentration of the medium (Arrondo et al., 1987). The appearance of a new α-helix substructure related to the E_2 conformation may indicate changes in the stalk and intramembranous helices that are involved in Ca^{2+} translocation.

The rearrangement of the structure induced by EGTA + vanadate results in a striking stabilization of the Ca^{2+}-ATPase against the high pressure (1500 - 2000 atm), compared with the extreme lability of systems containing only EGTA; less effective protection against high pressure was observed in the presence of 1-2 mM Ca^{2+} (Varga et al., 1986). These observations imply greater stability of the enzyme at high pressure in the E_1 or E_2 states than in the conformational equilibrium that prevails in a Ca^{2+}-free system in the absence of vanadate (Varga et al., 1986). The inhibition of pressure-induced denaturation of Ca^{2+}-ATPase by vanadate suggests that vanadate decreases the effective molecular volume of the Ca^{2+}-ATPase in the E_2-V state.

Tryptic cleavage of Ca^{2+}-ATPase as a conformational probe.

In sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle the primary cleavage of the Ca^{2+}-ATPase by trypsin at residue 514 (T_1 site) produces two large fragments (A and B) of nearly equal size (MacLennan and Reithmeier, 1982). Both fragments remain attached to the membrane with retention of ATPase activity and Ca^{2+} transport. The phosphate acceptor aspartyl residue is located in fragment A, while fragment B contains the lysine group that reacts with fluorescein-5'-isothiocyanate. Fragments A and B are structurally dependent on each other and dissociate only after denaturation (Rizzolo and Tanford, 1978). Secondary cleavage of fragment A by trypsin at the T_2 site produces the A_1 and A_2 subfragments with loss of Ca^{2+} transport, but the Ca^{2+}-stimulated ATPase activity is still retained (Scott and Shamoo, 1982).

The conformation of Ca^{2+}-ATPase exerts major influence upon its fragmentation by trypsin. Exposure of sarcoplasmic reticulum vesicles to 1-5 mM vanadate in the presence of EGTA has no effect on the tryptic cleavage of the enzyme at the T_1 site, but the subsequent hydrolysis of the A fragment into A_1 and A_2 subfragments at the T_2 site is completely blocked (Dux and Martonosi, 1983b). Therefore in the E_2 state the T_2 site is not accessible to tryptic cleavage. The cleaved Ca^{2+}-ATPase retains its ability to form two-dimensional E_2 type crystals.

Ca^{2+} or lanthanides, at concentrations sufficient to saturate the high affinity cation binding site of the Ca^{2+}-ATPase, overcame the

effects of vanadate and facilitated the cleavage of the Ca^{2+}-ATPase at the T_2 site, with inhibition of Ca^{2+} transport and loss of crystallization. Therefore in the E_1 state stabilized by Ca^{2+} or lanthanides, the T_2 site of Ca^{2+}-ATPase is accessible to tryptic cleavage.

Vanadate also inhibits the cleavage of the B fragment and similarly to its effect on the intact enzyme, it modulates the fluorescence response of fluorescein-5'-isothiocyanate covalently bound to the B fragment in the cleaved ATPase (Dux et al., 1985b). The retention of the fluorescence response of FITC after tryptic cleavage at the T_1 site implies that the A and B fragments interact and respond with concerted conformational changes to Ca^{2+} and vanadate, even without a covalent linkage between them.

Ca^{2+}-ATPase interactions in sarcoplasmic reticulum.

Interactions between lipoprotein complexes are common in biological membranes and contribute to the cooperative phenomena expressed in excitability, transport processes, receptor functions, and enzymatic activity. Evidence obtained by electron microscopic (Jílka et al., 1975; Franzini-Armstrong and Ferguson, 1985), ultracentrifuge (Møller et al., 1982), chromatographic (Andersen et al., 1986), chemical crosslinking (Chyn and Martonosi, 1977; Kosk-Kosicka et al., 1983; McIntosh and Ross, 1985; Keresztes et al., 1987), radiation inactivation (Vegh et al., 1968; Chamberlain et al., 1983; Hymel et al., 1984, 1985), and fluorescence energy transfer techniques (Vanderkooi et al., 1977; Papp et al., 1987; Highsmith and Cohen, 1987) indicates that the Ca^{2+}-ATPase of sarcoplasmic reticulum may also represent a self-associating system in which oligomers (dimers, tetramers) of the enzyme are present in equilibrium with monomers. The high concentration of Ca^{2+}-ATPase in the membrane (3-6 mM) is expected to promote such interactions. The clearest manifestation of such interactions is the crystallization of Ca^{2+}-ATPase, under appropriate conditions, in the native membrane.

The interactions can be monitored by Forster type fluorescence energy transfer between fluorescein-5'-isothiocyanate (FITC)-labeled donor- and eosin-5'-isothiocyanate (EITC)-labeled acceptor-ATPase molecules using the effect of acceptor fluorophore on the lifetime and intensity of donor fluorescence as an indicator (Papp et al., 1987). As alternative donor-acceptor pairs, N-iodoacetyl-N'(5 sulfo-1-naphthyl)ethylenediamine (IAEDANS) and iodoacetamidofluorescein (IAF) (Vanderkooi et al., 1977; Gingold et al., 1981; Watanabe and Inesi, 1982), or N-(1-pyrene)maleimide and N-7-(dimethyl)amino-4-methyl-3-coumarinyl maleimide (Yantorno et al., 1983) can also be used. The excimer fluorescence of pyrene covalently bound to the Ca^{2+}-ATPase (Papp et al., 1985, 1986) may monitor intramolecular, rather than intermolecular distances, and further work is needed to evaluate its usefulness (Ludi and Hasselbach, 1987; Martonosi, 1987).

The fluorescence energy transfer is not affected significantly by 0.2 mM EGTA + 0.2 mM vanadate that stabilizes the E_2 conformation, or by 0.4 mM Ca^{2+} that stabilizes the E_1 conformation of the Ca^{2+}-ATPase (Papp et al., 1987). Therefore the cyclic changes in enzyme conformation during Ca^{2+} transport are not likely to be associated with changes in the monomer-oligomer equilibrium of Ca^{2+}-ATPase. Changes in transmembrane potential were also without effect on the energy transfer (Papp et al., 1987), although potential-dependent changes in the conformation of Ca^{2+}-ATPase are known to occur (Jona and Martonosi, 1986).

The energy transfer is significantly reduced by low concentration of detergents that do not cause detectable solubilization of the membrane, and it is completely abolished by denaturation of Ca^{2+}-ATPase with urea or guanidine (Papp et al., 1987). These observations suggest that the energy transfer requires the native structure of the Ca^{2+}-ATPase and it is sensitive to the physical properties of the lipid environment. Although some contribution by non-associated ATPase molecules to the energy transfer is likely (Estep and Thompson, 1979; Champeil et al., 1982), much of the observed energy transfer appears to arise from genuine oligomers of Ca^{2+}-ATPase. Highsmith and Cohen (1987) suggest that Ca^{2+}-ATPase tetramer is the dominant oligomeric form.

Microcrystals of Ca^{2+}-ATPase in detergent-solubilized sarcoplasmic reticulum.

Two-dimensional microcrystalline arrays of Ca^{2+}-ATPase molecules develop in detergent-solubilized sarcoplasmic reticulum upon exposure to 20 mM Ca^{2+} for several weeks at 2°C, in a storage medium of 0.1 M KCl, 10 mM K-MOPS, pH 6.0, 3 mM $MgCl_2$, 3 mM NaN_3, 5 mM DTT, 25 IU/ml Trasylol, 2 μg/mg 1,6-di-tert-butyl-p-cresol, 20% glycerol and 2-4 mg detergent ($C_{12}E_8$ or Brij 36T) per mg protein (Dux et al., 1987). Preparations stored under these conditions retain significant Ca^{2+}-stimulated ATPase activity when assayed in the presence of azolectin. The stability of the enzyme is a necessary but not sufficient condition for crystallization, since the activity of solubilized Ca^{2+}-ATPase was preserved under a variety of conditions that did not lead to crystallization (Pikula et al., 1987).

The crystals consist of stacked lamellar arrays of Ca^{2+}-ATPase molecules. Analysis of electron micrographs obtained from edge-on views of these stacked sheets yield a sheet thickness of ≃ 180 Å and show lamellae with ATPase molecules extending out of both sides. The core of the lamellae probably contains a lipid-detergent phase into which the hydrophobic tail portions of ATPase molecules are symmetrically inserted (Mullner et al., 1987). There is no suggestion in these edge-on views of a regular pattern in the membrane stacking. In-plane projection of single sheets have been difficult to obtain, due to the strong tendency of the sheets to aggregate. Diffraction patterns, when obtained, have indicated that the unit cell is orthorhombic, with sides of 164.2 ± 2.2 and 55.5 ± 1.5 Å. Interaction between the hydrophilic head portions of the ATPase molecules are expected to play a role in the stacking of the lamellae. The high concentration of Ca^{2+} (20 mM) and the low pH (pH 6.0) may promote these interactions by minimizing charge repulsions. Under similar conditions Ca^{2+} also promoted the formation of E_1-type two-dimensional crystalline tubules in intact sarcoplasmic reticulum in the absence of detergents (Dux et al., 1987), and facilitated the crosslinking of Ca^{2+}-ATPase in intact and solubilized sarcoplasmic reticulum, with the formation of dimers, tetramers and higher oligomers (Keresztes et al., 1987). Experiments are in progress to generate three-dimensional crystal forms from fully delipidated Ca^{2+}-ATPase that are suitable for x-ray diffraction analysis using detergent-small amphiphile combinations (Michel, 1983; Deisenhofer et al., 1985).

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MEASUREMENT OF PERTINENT OXYGEN CONCENTRATIONS IN BIOLOGICAL SYSTEMS*

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INTRODUCTION

Throughout his illustrious career, Prof. Szent-Gyorgyi studied processes that involved redox metabolism and which, therefore, depended on oxygen concentrations. Recently, he was studying reactive intermediates whose production and biological actions were closely related to redox metabolism. Our mutual interests in free radical reactions, other aspects of redox metabolism and cancer led to the opportunities I had to interact with Professor Szent-Gyorgyi. In gratitude for these stimulating discussions, I am pleased to dedicate this summary of our current work on measurement of oxygen concentrations and related redox metabolism (1-13) to Professor Szent-Gyorgyi. In keeping with his spirit of inquiry, the summary presented here is an unfinished story, with much of the essential work still in progress. Nevertheless, I feel that the results and concepts described here provide a potentially powerful experimental and conceptual approach to many significant problems.

The title includes the term "pertinent oxygen concentrations"--why this unusual phraseology? The redox reactions of interest in biochemistry and physiology usually are related to the oxygen concentrations at the reaction sites: i.e., inside cells and tissues. Typically,

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however, the oxygen measurements that are made are of oxygen consumption or extracellular oxygen concentrations. Such measurements are made because of a lack of methods to make the measurements that are desired. The research described here is aimed at measuring oxygen and redox metabolism in "pertinent" locations, i.e., intracellularly and in functioning tissues.

Our experimental approach is based on the ability of magnetic resonance techniques to obtain data from biological systems in a nonperturbing manner. We use both electron spin resonance (ESR or EPR) and nuclear magnetic resonance (NMR) to observe oxygen directly and indirectly, exploiting the paramagnetism of oxygen and its effect on redox metabolism. Our experimental approaches involve the direct measurement of intracellular and extracellular oxygen by ESR, the measurement of oxygen concentrations in tissues by ESR imaging and feasibility studies aimed at the measurement of oxygen concentrations in vivo. The latter approach primarily uses NMR imaging with "contrast agents" whose concentration reflects oxygen concentrations but it also includes the observation of direct effects of oxygen by both NMR and ESR imaging techniques.

METHODOLOGY PRINCIPLES

Measurement of Concentrations of Intracellular Oxygen in Cell Suspensions

The basic physical phenomenon on which the measurements are based is the effect of oxygen on the ESR spectra of stable free radicals. The effects occur because oxygen is paramagnetic and therefore can influence the relaxation times of free radicals. These effects have been used to

measure oxygen concentrations because the extent of the effects are proportional to the concentration of oxygen.

The effects of oxygen on ESR spectra can be manifested by several different experimental parameters. For free radicals with superhyperfine structure the extent of the resolution of the hyperfine structures is inversely related to the oxygen concentration (Figure 1) so that empirical parameters relating the relative line heights of spectral components can be used to determine oxygen concentrations. Another approach is to use the effects on the line width of narrow hyperfine lines; this approach is especially suitable for perdeuterated nitroxides which have quite narrow lines (Figure 2). Figure 3 illustrates that the data from such experiments can provide a parameter than is a reflection of oxygen concentrations. Another approach is to use the effects of oxygen on the microwave power saturation of stable free radicals such as nitroxides or melanin. The power saturation effects are measured by determining the "$P_{1/2}$" or microwave power at which the peak height is half that which would occur in the absence of saturation. The presence of oxygen diminishes power saturation and this effect is proportional to the concentration of oxygen.

The second key to the measurement of intracellular oxygen concentrations is to study selectively the ESR spectra of free radicals within the cell. Several approaches are possible. The simplest approach, which is illustrated in Figure 2, is to add relatively large concentrations of an ionic paramagnetic species, usually a paramagnetic metal ion. In high concentrations these substances broaden out the ESR spectra of the free radicals they approach to such an extent that the ESR spectra of the free radicals are essentially not visible. Because these metal ions are

charged they do not cross cell membranes readily and therefore affect only the extracellular free radicals, so the ESR spectra of the intracellular free radicals remain observable. Another approach is to use a free radical that selectively concentrates in the cell. This can be done in cell suspensions with the use of highly lipophilic free radicals which will partition into the membranes and other lipid rich areas of the cell. Selective intracellular localization of the free radicals also can be achieved by the use of free radicals whose chemico-physical nature results in their unidirectional movement into cells: we have achieved this in two different ways, by having the free radicals in a form that is phagocytized by the cells and by using free radicals that become converted intracellularly into charged species by the hydrolysis of an ester bond.

Using some of the approaches outlined above it also is possible, in principle, to obtain information on oxygen concentrations in particular parts of the interior of cells. For example, lipophilic stable free radicals will be found primarily in membranes while aggregates that are phagocytized are likely to be located in lysosomes.

Measurement of Concentrations of Extracellular Oxygen in Cell Suspensions

These studies use the same physical principles used for measurements of concentrations of intracellular oxygen except that the spectra due to the intracellular free radicals are suppressed relative to those due to extracellular free radicals. One approach is to use a nitroxide that is fully charged at physiological pH and does not enter cells appreciably and which also has a spectral parameter that is sensitive to oxygen. The nitroxide "Cat_1" (Fig. 1) meets these criteria fully, being a cation at physiological pH and having oxygen sensitive superhyperfine structure. A

second approach is to use a stable free radical that distributes throughout the system, has an appropriate oxygen sensitive parameter and to limit the amount of intracellular volume in the system to less than 10%. Under these circumstances, the spectra from the extracellular free radicals predominate; we have found perdeuterated Tempone (Figure 2) to be satisfactory for this approach.

Measurements of Oxygen Concentrations in Tissues in vitro and in vivo by ESR

The methods used for cell suspensions can be extended to tissue samples and, most probably, to intact animals by using ESR imaging techniques. The basic approaches to ESR imaging have been described recently (12-18); they have been applied successfully primarily in model systems and in vitro but it seems likely that comparable sensitivity can be obtained in vivo. Using model systems, we have used three different approaches to obtain ESR images that reflect oxygen concentrations (101).

One approach is to use the line broadening effect of oxygen: the paramagnetic nature of oxygen will broaden ESR spectra of appropriate free radicals so if the images are based on the intensity of the line height, areas with equal concentrations of nitroxides but different oxygen concentrations will have different intensities in the ESR image.

A second approach is to use the effect of oxygen on the power saturation of the free radicals: the presence of oxygen will increase the microwave power required to obtain partial saturation of the ESR spectra. The most general method is to obtain images at several different microwave powers and then to construct an image based on differences of intensity between normalized spectra obtained at a non-saturating (low) microwave power and spectra obtained at higher microwave

powers at which saturation occurs. The resulting image will have brightness that is proportional to the oxygen concentration of the area. We have used both nitroxides and melanin as the free radicals for this type of study.

A third approach is to use the oxygen dependent metabolism of nitroxides as a means to image areas with low oxygen concentrations. Nitroxides are potential NMR contrast agents whose presence effects NMR images by altering relation times of protons due to effects of their magnetic moment on that of the protons (19-21). We have shown recently that the rate of reduction of some nitroxides to the non-paramagnetic hydroxylamines is much faster in cells with very low oxygen concentrations (2,3,9). Consequently in such regions the nitroxide concentration will decrease rapidly and these regions will have less intensity in the ESR image.

Measurements of Oxygen Concentrations in Tissues in vitro and in vivo by NMR

We have used two different approaches aimed at obtaining oxygen dependent NMR images. Our results so far indicate that both approaches should be feasible but we are just beginning to carry out the NMR imaging studies themselves. An especially attractive aspect of these approaches is that NMR imaging techniques have developed rapidly into being both very powerful and available at many places. Our approaches use the effects of paramagnetic substances (often termed "contrast agents") on proton relaxation times because relaxation times are the principal parameters used to obtain contrast in NMR images (2,4,8,22).

One approach is to use the effect of oxygen itself. Oxygen is paramagnetic and therefore capable of increasing the rate of relaxation of

protons. It has been established for some time that this effect occurs but is relatively weak. Although the magnitude of the effect of oxygen on the relaxation of water protons is likely to be undetectable by current NMR imaging approaches, this may not be the case for effects on relaxation of the protons of lipids. The effect is greater on lipid protons because of the four-fold greater solubility of oxygen in lipids and the higher viscosity of lipid-rich systems. We have explored the feasibility of making measurements in vivo by determining the extent of the effect of oxygen on the relaxation rates of lipids in vitro (8).

The second approach is to use the paramagnetic nitroxides as contrast agents for NMR imaging and to select conditions such that the concentration of these contrast agents will reflect the local oxygen concentrations due to the metabolic conversion of nitroxides to the non-paramagnetic hydroxylamines (2,7). The effectiveness of nitroxides as contrast agents for NMR has been demonstrated by Brasch and colleagues (19-21). We seek to determine the feasibility of our approach by obtaining quantitative data on: 1) the oxygen dependent rate of conversion of nitroxides to the non-paramagnetic hydroxylamines and 2) the effectiveness of nitroxides in enhancing the relaxation of protons. For the former measurement we use cell suspensions and using ESR measure changes in the concentrations of nitroxides under different oxygen concentrations. For the measurements of relaxivity we use the NMRD technique developed by Koenig and Brown (4,23,24).

RESULTS AND DISCUSSION

Illustrative results of the approaches described in the Methodology section are shown here as figures. For more complete descriptions of the techniques the reader is referred to the original articles indicated in the figure legends.

The method to measure intracellular oxygen is shown schematically in Figure 2. Figure 3 shows that this technique provides data at concentrations of oxygen that are pertinent to studies of cells under physiological conditions (the concentration of oxygen in air is 210 μM). In recent studies in our laboratory we have found that at high rates of oxygen utilization in some cellular systems, a clear difference exists between the concentrations of oxygen in the intracellular and extracellular compartments; this finding indicates the value of making measurements of intracellular oxygen concentrations rather than the usual measurements of extracellular concentrations of oxygen. As indicated later in this section the ability to measure intracellular oxygen concentrations in cell suspensions may be important in developing and understanding techniques to measure oxygen concentrations in vivo by magnetic resonance imaging, in order to calibrate the oxygen dependent rate of reduction of the image modifying agents (nitroxides) used to obtain the images. The results of the studies to measure intracellular oxygen in cell suspensions indicate that this is a practical and useful way to obtain data on this important physiological and pathophysiological parameter. In addition to providing data needed to relate normal cellular functions to the pertinent oxygen concentrations this technique may play a significant role in the understanding of damage due to "oxygen radicals," "oxidative stress," and related phenomena.

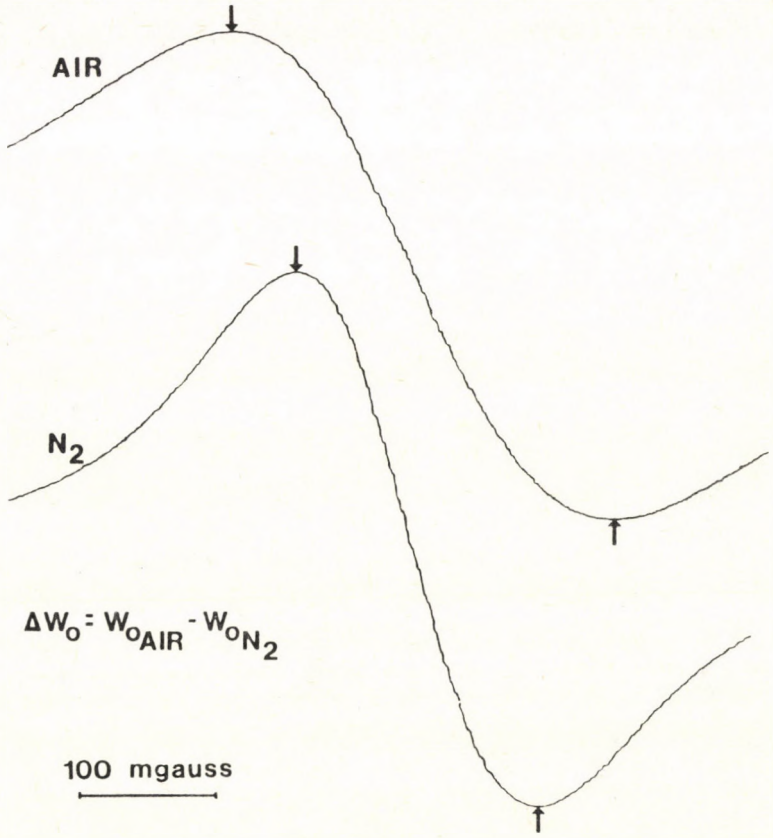

Figure 1. Method to Measure Oxygen by Effect on Superhyperfine Structure of Spectra of Nitroxides. The spectra of the midfield line of the stable free radical, the nitroxide "Cat_1" (chemical formula shown), in the presence (lower spectrum) and absence (upper spectrum) of oxygen indicate that oxygen can affect the resolution of the superhyperfine lines. An empirical parameter ("C") of the concentration of oxygen can be calculated as C = (A + B)/h and it is found that in the range of oxygen concentrations of interest C varies linearly with the concentration of oxygen. If the nitroxide used for such measurements is fully charged, as is the case for the nitroxide illustrated in this figure, then it will stay extracellularly and can be used to measure extracellular oxygen concentrations. (Reproduced with permission, from reference 10; please refer to the reference for additional experimental details.)

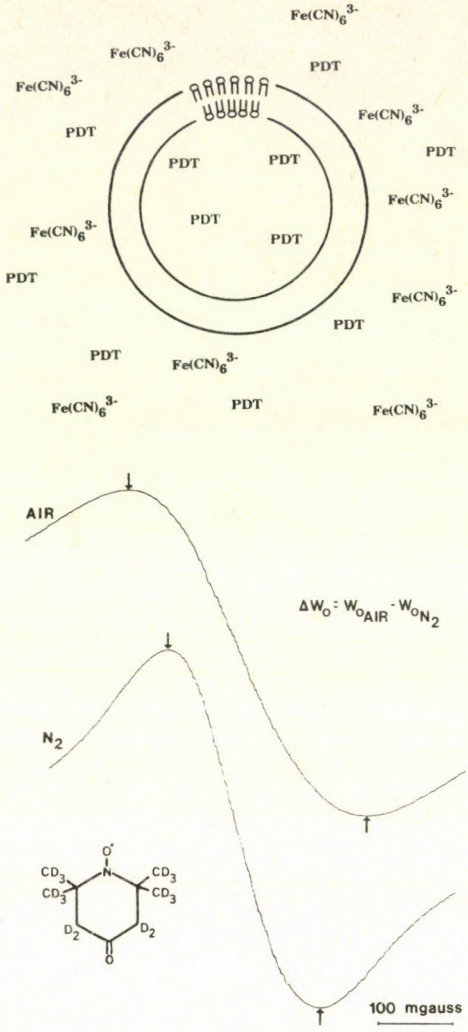

Figure 2. Method of measuring intracellular oxygen. (A) Schematic representation of a cell, nitroxides (PDT) and broadening agents ($Fe(CN)_6^{3-}$, ferricyanide). The broadening agent effectively eliminates the ESR spectra of extracellular nitroxides. (B) ESR spectra used to measure concentrations of intracellular oxygen: spectra of 0.10 mM PDT (chemical formula shown) in the presence (a) and absence (b) of air. The peak-to-peak linewidths are measured as shown by the small arrows. The difference, ΔW_O, changes linearly with oxygen concentration. Calibrations are made in the media in which the oxygen concentration is measured. (Reproduced with permission, from reference 10; please refer to the reference for additional experimental details.)

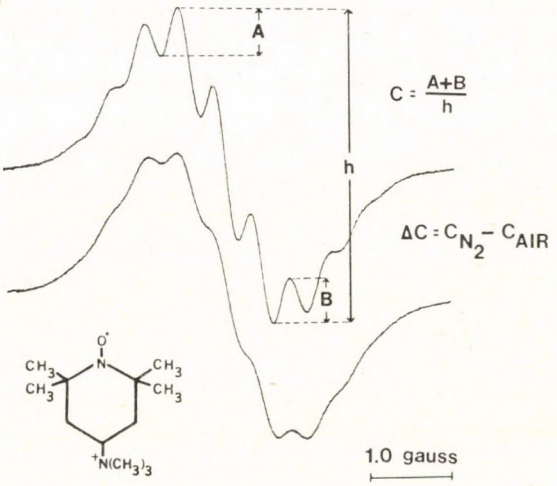

Figure 3. Relationship Between Oxygen Concentrations and the ESR Measurement Parameter ΔW. The parameter ΔW is the increase of linewidth of the nitroxide perdeuterated Tempone or "PDT" (4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl) in a cellular suspension at 37°C as a function of oxygen concentration. The data fit the linear regression equation: $\Delta W_o = 3.82 \times 10^{-1} [O_2]^2 + .385 [O_2] - 0.00195$; r = 0.998. (Reproduced with permission, from reference 5; please refer to the reference for additional experimental details.)

Figures 4-6 illustrate how we may be able to extend measurements of oxygen concentrations into more complex systems such as tissues and eventually, to intact animals, by means of ESR imaging. Figure four illustrates oxygen dependent differences observed in an ESR image based on solutions of nitroxides in a gas permeable tube. The effect is seen most clearly by subtracting the images obtained under the different oxygen concentrations used in the illustrated experiment, using the invariant images in the gas impermeable glass tubes for normalization of the images. The physical basis for the observed effect is the oxygen induced broadening of the ESR spectra of the nitroxide. The direct use of this approach in tissues would require the presence in the imaging field of a calibration standard.

An alternative approach is illustrated in Figure 5. In this illustration two capillaries containing the same nitroxide, but having 100% oxygen or nitrogen as the gas in the sample tube, were imaged at two different microwave powers. At the higher microwave power the sample equilibrated with oxygen had a stronger signal because the presence of oxygen allowed more rapid relaxation of the nitroxide thereby decreasing the amount of power saturation that occurred. At lower microwave powers there would be less power saturation and therefore by making measurements at two or more microwave powers it should be fairly straightforward to determine oxygen concentrations by this approach.

The third approach is to use the oxygen dependent cellular metabolism of nitroxides. Two sample tubes containing viable mammalian cells and the nitroxide 5-doxyl stearate were imaged first with both samples equilibrated with air and then again after one of the tubes was perfused with nitrogen for seven minutes before returning to air

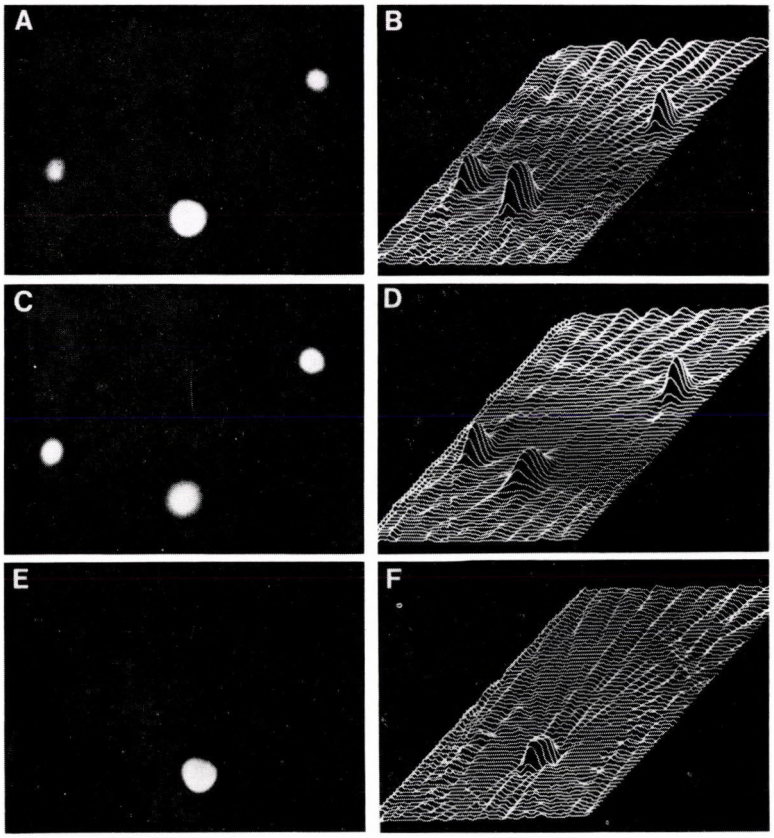

Fig. 4. ESR Images Reflecting Differences in Oxygen Concentrations — Based on Oxygen-Induced Line Broadening. An ESR imaging device operating at 9 GHz was used to obtain images of three capillary tubes containing the nitroxide ^{15}N PDT in which the ^{14}N nucleus was replaced with ^{15}N in order to obtain a more narrow and intense spectrum for imaging. The top two glass capillary tubes (i.d. = 0.63 mm) were sealed in air. The bottom capillary was made of thin teflon (i.d. = 0.81 mm) and was permeable to gases. A, C, E are spin density plots and B, D, F are contour plots. The gas conditions were: A, B nitrogen flowing; C, D oxygen flowing. E, F are the results of subtracting the data of C from those of A; they reflect the fact that the intensity of the image of the gas permeable capillary tube decreased when oxygen was flowing because the lines of the ESR spectra became broader due to the effects of the paramagnetic oxygen molecule. (Reproduced with permission, from reference 12; please refer to the reference for additional experimental details.)

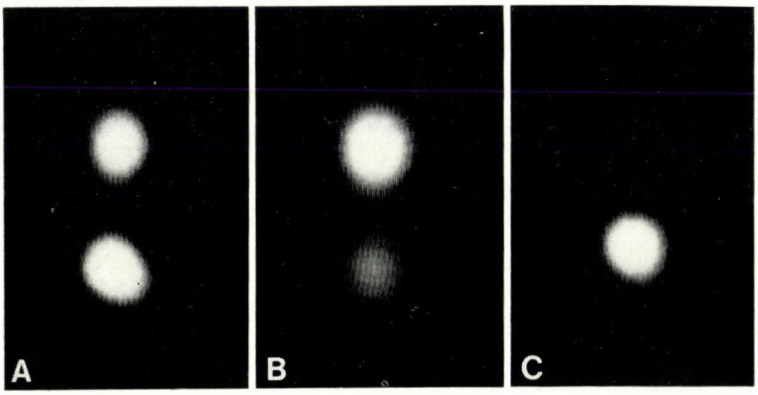

Fig. 5. ESR Cross-Sectional Images Reflecting Differences in Oxygen Concentrations — Based on Oxygen-Induced Changes in Microwave Power Saturation. The upper sample tube was made of glass (i.d. = 0.63 mm) and was sealed in air, the lower sample tube was made of gas permeable Teflon (i.d. = 0.81 mm) and was perfused with 100% nitrogen during the experiment; both contained 1 mM Cat_1. The incident 9.5 GHz microwave power was: 5 mW (A) or 15 mW (B). (C) is the image obtained by subtracting (B) from (A). At the higher microwave power the nitroxide in the oxygen containing tube had a relatively higher signal intensity because the paramagnetic oxygen increased the relaxation rate of the nitroxide thereby decreasing the amount of power saturation. Such differences in the power saturation of free radicals in different locations would be detectable *in vivo* independent of differences in the amount of free radicals in the different locations. (Reproduced with permission, from reference 12; please refer to the reference for additional experimental details.)

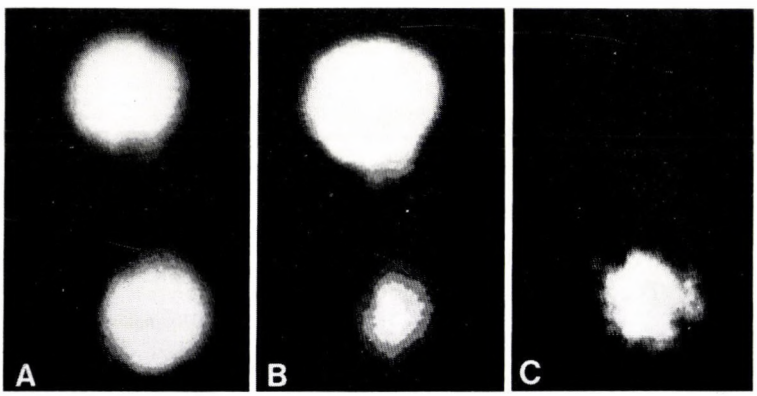

Fig. 6. ESR Images Reflecting Differences in Oxygen Concentrations — Based on Oxygen-Dependent Differential Rate of Metabolism of Nitroxides by Mammalian Cells. The samples were placed in gas permeable Teflon tubes (i.d. = 0.81 mm) and contained mouse thymus-bone marrow cells and the nitroxide 5-doxyl stearate. The samples first were imaged while both were perfused with air (A) and then imaged again in air after the bottom sample tube was perfused with 100% nitrogen for seven minutes (B). The image obtained by subtracting image (B) from image(A) indicates the faster rate of reduction of the nitroxide to the non-paramagnetic hydroxylamine that occurs in cells that are deficient inoxygen. Such an image can indicate areas of hypoxic cells. (Reproduced with permission, from reference 12; please refer to the reference for additional experimental details.)

perfusion. During the time that the cells were in an oxygen poor environment the rate of metabolism of the nitroxide to the nonparamagnetic hydroxylamine occurred at a much more rapid rate (3,7,9). Consequently the image from that sample became less intense relative to the sample that was well oxygenated for the entire time of the experiment. In tissues and in intact animals this same phenomenon should be able to be applied to detect differences in oxygen concentrations. In the more complex situation in real tissues or in vivo, it probably will be desirable to obtain several sets of data at different times after administering the contrast agents in order to determine the rate of loss of intensity of the ESR signal.

Figures 7 and 8 illustrate how the overall approach could be extended to take advantage of the rapid development of NMR imaging. Figure 7 indicates the difference in relaxation rates that occur in model lipids under different concentrations of oxygen. The differences are larger in lipids than in aqueous solutions because oxygen is four-fold more soluble in lipids than in aqueous solutions and also because of the greater viscosity of lipids. As a consequence of these factors, 100% oxygen increases the relaxation rates of lipid protons by about 2 sec^{-1}. An effect of this magnitude may be detectable directly by existing NMR imaging devices and it should be possible to design new imaging devices that would be more sensitive to such effects.

The principles of the use of nitroxides as metabolically responsive contrast agents for in vivo NMR techniques, including NMR imaging (often termed MRI) have been described briefly in the methods section. One of the requirements for successful use of this approach is the development of nitroxide contrast agents whose concentration reflects a metabolic

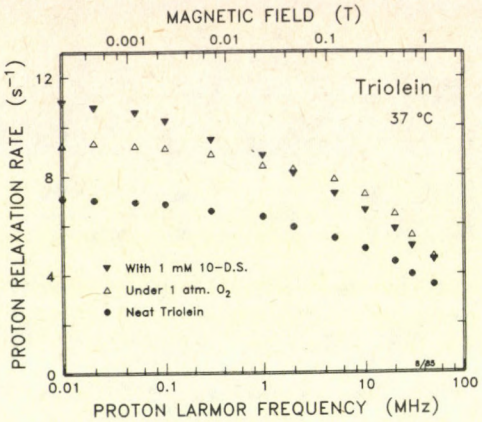

Figure 7. The Effect of Oxygen on Relaxation of Lipid Protons. Relaxation rates were determined in the instrument developed by Koenig and Brown for measurements of spin-lattice relaxation rates of protons (23) in pure lipids (triangles = triolean, squares = linoleate) at different resonant magnetic fields. The dotted line indicates the oxygen-induced fractional increase in relaxation rates ($1/T_1$) of protons of triolein. Most clinical NMR imaging instruments operate at magnetic fields of 0.1 to 1.5 Tesla; such changes in relaxation rates should be detectable in optimized clinical imaging instruments. (Reproduced with permission, from reference 8; please refer to the reference for additional experimental details.)

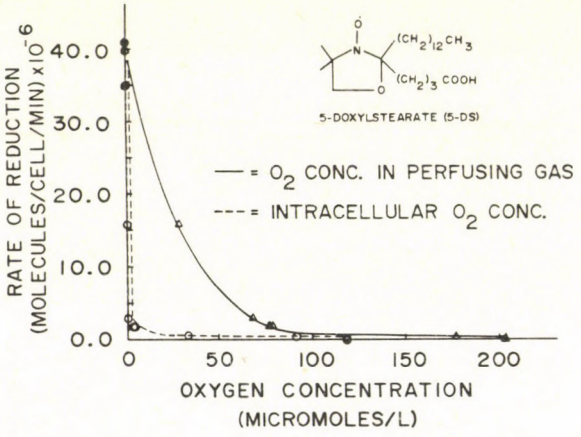

Figure 8. The Effects of the Concentration of Oxygen on the Rate of Reduction of Potential NMR Imaging Contrast Agents. The data were obtained by ESR measurements of the change in concentration of the nitroxide as a function of oxygen concentrations, in a suspension of mammalian cells at 37°C in a gas permeable sample tube. Relationships are shown for both the concentration of oxygen perfusing around the sample tube (solid line) and the intracellular oxygen concentration as measured by the techniques described in Figures 1-3. (Reproduced with permission, from reference 7; please refer to the reference for additional experimental details.)

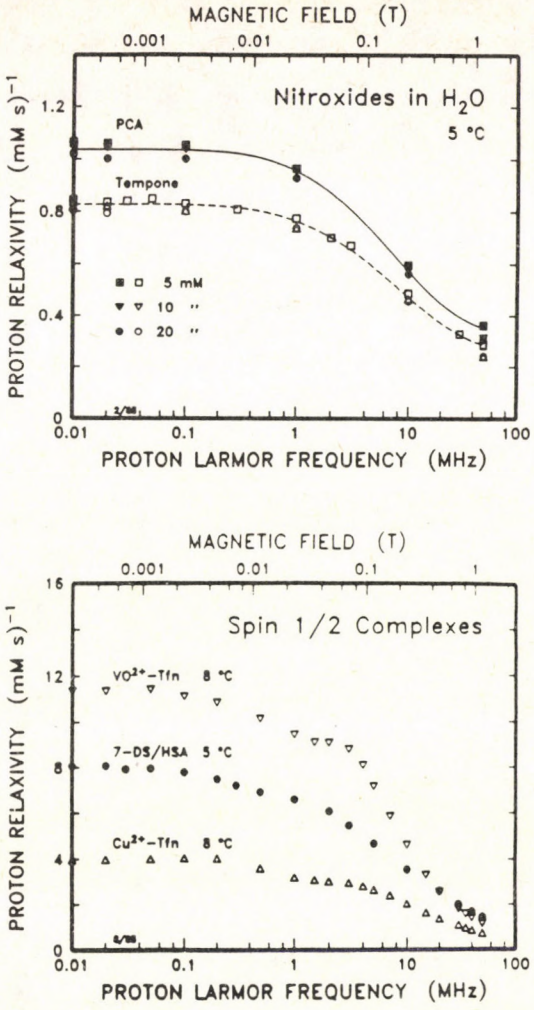

Figure 9. Efficacy of Nitroxides as Relaxers of Protons. The upper figure indicates the relaxivity induced by aqueous solutions of two nitroxides. The bottom figure indicates the relaxivity of three different protein bound paramagnetic species; the middle curve is that for the nitroxide 7-doxyl stearate complexed with human serum albumin (the other two curves are for metal ions on transferrin). The relaxivity of a nitroxide, when it is complexed with a macromolecule, increases to a level simjlar to that of similarly bound paramagnetic metal ions; this effect is due to the slower rotation of the bound nitroxide, which makes it a more efficient relaxer of protons. (Reproduced with permission, from reference 4; please refer to the reference for additional experimental details.)

parameter such as hypoxia; Figure 8 demonstrates that several nitroxides have this property and there are now a number of studies that indicate the generality of this phenomenon. That we can also meet the second requirement, that nitroxides be effective relaxers, is indicated in Figure 9. We see that while nitroxides are only modestly effective relaxers in aqueous solutions (Figure 9A), when they are bound to macromolecules they can be as effective relaxers as similarly bound paramagnetic metal ions (Figure 9B).

ACKNOWLEDGEMENTS

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ACTOMYOSIN ATPase AND SUPERPRECIPITATION: THEIR MODIFICATION BY RUTIN *

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Rutin markedly accelerates the superprecipitation of rabbit skeletal muscle myosin B, in accord with the effect of its aglycon, quercetin, on the tension development of glycerinated fibers of rabbit skeletal muscle (Kurebayashi and Ogawa, 1985). This accelerating effect on superprecipitation can also be observed with desensitized myosin B. However, rutin exerts much less accelerating effect on the ATPase activity of myosin B and no definite effect on that of desensitized myosin B.

These results are interpreted that rutin enhances the availability of chemical energy of ATP for mechanical performance, perhaps by repressing inherent inhibitory mechanism.

* Dedicated to the memory of Professor Albert Szent-Györgyi, whom my scientific carrier has deeply been indebted to.

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INTRODUCTION

At the beginning of 1940's, Albert Szent-Györgyi and his colleagues have made epoch-making discoveries in muscle research and established one of the most successful concepts in biological sciences, i.e., the interaction of myosin and actin in the presence of ATP under appropriate conditions represents the fundamental mechanism underlying muscle contraction. This interaction is characterized by two distinct features: one is a chemical expression, viz., ATP breakdown, and the other a physical expression, viz., contraction or superprecipitation (15,16).

In 1941, Szent-Györgyi observed that vigorous contraction of the thread made of myosin B, or natural actomyosin, was induced by ATP (13). This finding was noted by him as the most impressive observation in his research life (17). A little later an interesting phenomenon, termed superprecipitation, drew his attention (14). In his original article he pertinently described this phenomenon as the shrinkage of actomyosin particles (14). Later his emphasis was laid on the shortening of plug-like precipitates, so some people understood rather erroneously that the plug formation was the essential step of superprecipitation. Whatever the definition of superprecipitation might be, Szent-Györgyi has clearly concluded that thread contraction and superprecipitation are identical in nature.

Szent-Györgyi himself placed emphasis on the physical aspects of the myosin-actin-ATP interaction, viz., superpre-

cipitation or contraction of thread. Followers of Szent-Györgyi, however, have preferred the ATPase activity as representing muscle contraction. This was partly due to the difficulty in expressing such physical changes in a quantitative manner, but it was also supported by the general current of biological sciences to consider that chemical approaches should be direct access to the essence of the matter. Furthermore, the success in preparing H-meromyosin S-1 (9) has enabled people to deal with the actomyosin system in a soluble state, in which no apparent physical change is appreciable, and has given an impression to many muscle researchers that they are dealing with genuine molecular mechanism of muscle contraction (we must be aware of the fact, however, that we are dealing with actin only in its filamentous state, not in its single-molecular state). In this way, it has become a common understanding of many scientists that actomyosin ATPase is almost the synonym of muscle contraction.

There is no doubt that ATP breakdown is inseparably associated with contractile processes and ATP is sole energy source for bringing about contraction. However, so far no experimental evidence has been presented for the assumption that contraction is a simple function of the ATPase activity and that the ATPase activity is the direct chemical expression of muscle contraction.

In this respect, Endo and Kitazawa (4) have shown that caffeine of a high concentration enhances the tension developed by glycerinated fibers of Xenopus cardiac muscle at low Ca^{2+} concentrations. Further, Kurebayashi and Ogawa (7) have

recently reported that quercetin at a much lower concentration exhibits a similar but more marked effect on glycerinated fibers of rabbit fast skeletal muscle.

This has raised an interesting question whether the ATPase is also accelerated in parallel with increase in the tension development by quercetin. However, it is not an easy task to make quantitative determination of the ATPase activity of fiber models.

In 1961 Ebashi (1) has presented a view that the increase in the turbidity of suspended actomyosin gel can represent the shrinkage of each particulate of actomyosin gel, the primary step of superprecipitation. By using diluted suspension of actomyosin, he avoided the precipitation of bulky aggregates of actomyosin gel, or plug formation; "superprecipitated" actomyosin gel particles remained suspended for hours. This enabled him to describe the superprecipitation in a quantitative manner by simply measuring the increase in absorbance. His later work including the studies on troponin was made mainly by the use of this procedure (2, 3).

Recently, the use of superprecipitation as a tool for studying muscle contraction is further supported by the report of Kohama et al. (6) that various properties exhibited by superprecipitation are more akin to those of the contraction of fiber models such as glycerinated fibers or skinned fibers than are those of ATPase activities. On this view, it may be worthwhile to examine whether or not the effects of quercetin demonstrated with glycerinated fibers can be reproduced with superprecipitation, with which the ATPase activity can easily

be determined. If this is so, it will provide us the means to answer to above-mentioned question whether the ATPase activity is simply related to contraction.

Since quercetin is almost insoluble in water, it disturbs the measurement of turbidity change. Consequently, rutin, a soluble glycoside of quercetin, was used in this study. Using this drug, an interesting relationship between the contractile processes and ATPase activities has been presented.

MATERIALS AND METHODS

Myosin B was prepared from rabbit leg muscle by the routine procedure of our laboratory (2). Desensitization of myosin B was carried out as described in a previous paper (3). Superprecipitation experiment was carried out by the method of Ebashi (1). The ATPase activity was determined by measuring liberated inorganic phosphate using Malachite Green (5).

RESULTS

In Fig. 1 the effects of rutin on the relationships between the Ca^{2+} concentration and the ATPase activity or superprecipitation of myosin B are presented. Rutin enhances both the actomyosin ATPase activity and the superprecipitation, but the degree of enhancement of the former is much less than that of the latter.

One might argue, however, that the degree of superprecipitation expressed by the procedure described in the legend to Fig. 1 does not necessarily indicate the linear correlation with the actomyosin activation; in other words, the degree of

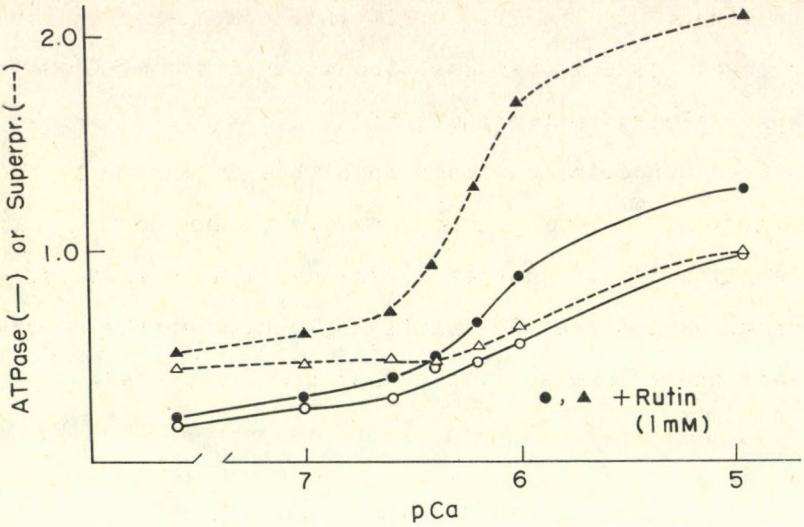

Fig. 1 Effects of rutin on relationships between pCa and superprecipitation or ATPase activity of myosin B

The ATPase activity at pCa 4.95 in the absence of rutin was taken as a unity and other activities were expressed relative to this value. The activity was determined in the solution containing 0.03M KCl, 1mM Mg, 0.02M imidazole buffer (pH 6.8), 0.04 0.06mg/ml of myosin B and 0.5mM ATP at 25°C. The unity corresponds to 0.4μmole Pi/min·mg protein. The unity of the degree of superprecipitation was the value of absorbance at 2 min after addition of ATP in the absence of rutin under the same conditions as above but at 21°C.

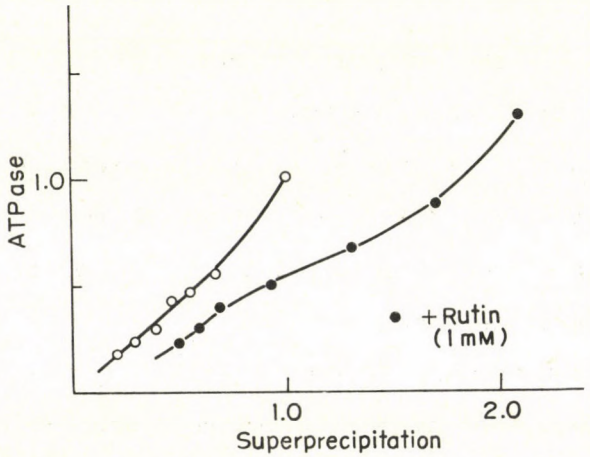

Fig. 2 Effects of rutin on relationships between superprecipitation and ATPase activity of myosin B

The data shown in Fig. 1 were replotted. Note that more marked superprecipitation was induced by rutin at the same ATPase level.

activation exhibited by superprecipitation might be exaggerated. Hence, all the results are replotted in Fig. 2, where the relationship between the ATPase activity and superprecipitation is shown. It is clear that rutin makes this relationship much sharper. This means that in the presence of rutin, much lower ATPase activity is enough to produce the same degree of superprecipitation, i.e., rutin can make the conversion of chemical energy into mechanical work more efficient. We cannot deny the possibility that the results shown in these figures would involve something related to Ca^{2+}, for instances, (i) rutin would elevate the affinity of troponin for Ca^{2+}, and/or (ii) it would make the coupling between the Ca-troponin-tropomyosin and the actomyosin systems more tight. Kurebayashi and Ogawa (7) have already shown that quercetin does not elevate the affinity of glycerinated fibers for Ca^{2+}. However, they used the contractile system associated with the troponin system, so their experiment did not eliminate the second possibility.

The advantage of myosin B is that it can be "desensitized", i.e., its troponin system can be removed rather easily. Figs. 3 and 4 show the results obtained by the use of desensitized myosin B. It is shown that rutin has essentially the same effect on desensitized actomyosin system as above. It certainly enhances the superprecipitation, but virtually no effect on ATPase activities; it rather represses the activity at lower ionic strengths. Thus the enhancement of contractility by rutin has nothing to do with Ca^{2+}-related regulatory mechanism, but is directly concerned with the essential proc-

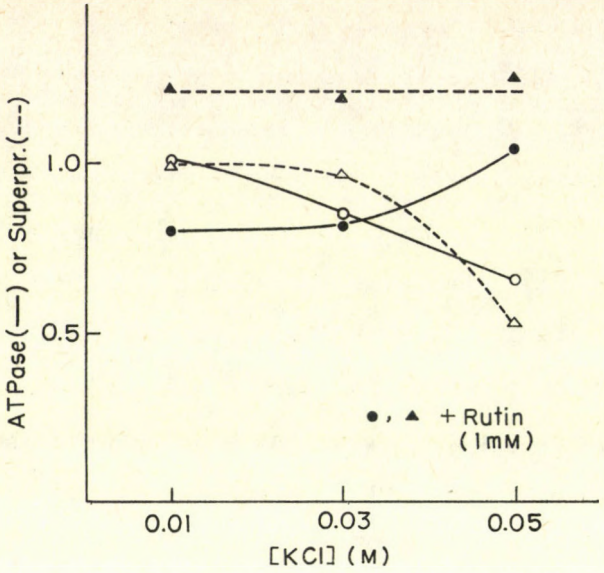

Fig. 3 Effects of rutin on superprecipitation or ATPase activity of desensitized myosin B at different ionic strengths
Experimental conditions were essentially the same as those in Fig. 1 except KCl concentrations (in this series, a fairly marked increase in the ATPase activity was observed at 0.05M KCl, but not in other cases).

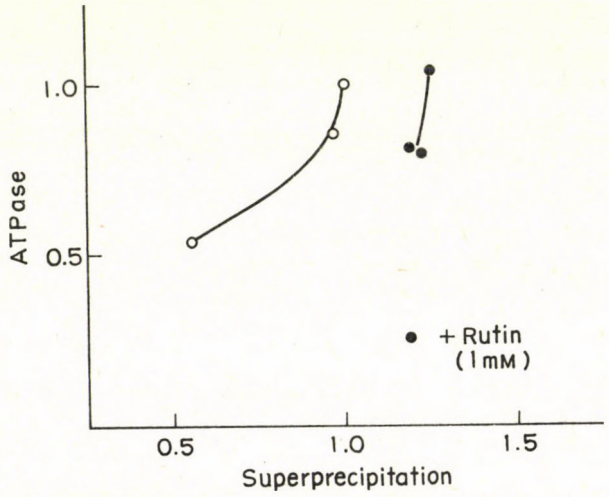

Fig. 4 Effects of rutin on relationships between superprecipitation and ATPase of desensitized myosin B
The data shown in Fig. 3 were replotted. Note that effects of rutin were comparable to those shown in Fig. 2, indicating that Ca regulation system did not play its role in this phenomenon.

esses of the actomyosin-ATP interaction.

DISCUSSION

The results presented in this article have indicated that the relationship between the ATPase activity and superprecipitation can be modulated by some procedure. Since the relationship between pCa and superprecipitation shown in this article is essentially the same as that between pCa and tention development (7), and the superprecipitation is a model for in vitro contraction more akin to the contraction of fiber models than is the ATPase activity (6), these results can be considered as being equivalent to those demonstrated by muscle fiber models (4,7). In other words, this report using actomyosin suspension has confirmed these previous results. Furthermore, it has revealed that the modulation has nothing to do with Ca regulation system but is deeply involved in the myosin-actin-ATP interaction itself.

Discrepancy between contraction and ATP breakdown is not a new finding but has already been indicated by the experiments with living fibers or fiber models (8,10,12). Perhaps the first report along this line is the famous "Solandt's effect" (11). These results had tacitly been ascribed to mechanical hindrance due to compact structure of muscle fibers, but it is worthy of note that the suspension of actomyosin gel of loose structure also exhibits a discrepancy between these two activities.

Important conclusions may be deduced from these results: (i) The efficiency of conversion of the chemical energy derived from ATP breakdown into the mechanical energy for contraction can be modulated; (ii) Since this effect cannot be observed with aged preparation (the behavior of aged preparation is similar to that of the fresh preparation with rutin rather than to that without rutin; data not shown), the native actomyosin system seems to have some inherent inhibitory mechanism, which could be released by such an exogenous agent as rutin or caffeine.

At present we do not know the essential nature of this inhibitory mechanism and its physiological significance. From pharmacological points of view, however, the presence of such a mechanism has opened up the possibility of creating a drug to intensify the contraction by direct modulation of the actomyosin system, especially, in the case of cardiac muscle where the physiological contraction cycle is carried out in the range of Ca^{2+} concentration which does not produce maximum level of contraction.

Finally, references should be made to the matter concerning superprecipitation. There have been presented some criticism at the superprecipitation method. One is that this method often gives unreasonable results which are occasionally opposite to those expected from those of ATPase measurement. The answer to this criticism is rather simple: such results are not due to the nature of superprecipitation but due to the failure in optical arrangement. Since the light scattering of the suspension of actomyosin gel, each particulate of which

the diameter is 1-4um, does not follow Rayleigh scattering but Mie scattering, where the forward scattering is so strong that the light axis must be exactly arranged to prevent the contamination of undesirable scattered light.

The other is the claim that everyone can understand the nature of ATPase, but not that of superprecipitation. This is rather unreasonable comment. As shown in this article, the position of ATPase in the contractile processes is complicated. Furthermore, recent discovery of Yanagida et al.(18) has compelled us to change our conventional view as to "ATPase". In other words we have not yet understood the true nature of the ATPase activity.

The common subject of muscle research is nothing but contraction. Since superprecipitation is a good model of in vitro contraction (6), the discrepancy between superprecipitation and ATPase activity described in this article is relevant to the subject how chemical energy is converted into mechanical performance.

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Location of the TnI Binding Site in the Primary Structure of Actin*

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The binding of troponin I (TnI) to actin has been regarded as an important step in the inhibition of myosin activation by the actin-tropomyosin-troponin filament. In this work we have used the TnI-actin complex to identify the region of the actin sequence involved in interaction with TnI. Actin labeled at Cys-374 with 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin was crosslinked to TnI by incubation of the 1:1 TnI-actin complex with 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide. Uncrosslinked actin and the crosslinked actin-TnI complex were separated on an SDS-polyacrylamide gel and the slices containing the proteins were subjected to proteolysis with CNBr, 2-[(2-nitrophenyl)sulfenyl]-3-bromoindolenine, and hydroxylamine at Met-X, Trp-X and Asn-Gly bonds, respectively (Sutoh, Biochemistry 21,3654,1982). Comparison of the digestion patterns of actin and the actin-TnI complex indicates that TnI has been crosslinked to the N-terminal 12 residue fragment of actin. Although myosin subfragment 1 binds to the same segment (cf. Sutoh, Biochemistry 25,435,1986), it does not displace TnI from the actin filament as revealed by high speed centrifugation.

The calcium dependent regulation of skeletal muscle contraction involves tropomyosin and troponin, components with actin of the thin filaments (for recent reviews see Leavis and Gergely 1984, El-Saleh et al. 1986). At Ca^{2+} concentrations <0.1 μM the tropomyosin-troponin complex prevents activation of myosin ATPase by actin and force development by the muscle. Troponin contains three subunits, TnC, TnT and TnI, each having a specific function. TnC is the Ca^{2+}-binding subunit and TnT is regarded as the main link between the troponin complex and tropomyosin. TnI is able to inhibit the actomyosin ATPase even in the absence of the other subunits and of tropomyosin, but this inhibition is not Ca^{2+} sensitive, while Ca^{2+} sensitivity is a hallmark of the regulatory system in vivo. While the inhibition by TnI alone affects only one actin monomer per TnI, in the intact or the reconstituted filament one Tn complex controls, via its attached tropomyosin moiety, at least seven actin monomers. Actually, because of interactions among the tropomyosin molecules, the cooperativity extends to nearby actin molecules assembled with neighboring Tm-Tn units (Hill et al. 1980). For nearly fifteen years there has been evidence

In memoriam Albert Szent-Györgyi

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suggesting that Ca^{2+}-binding to troponin causes a release of TnI from a site involving actin and tropomyosin and in a not fully understood way leads to activation of the actin-myosin site. This view has gained recent support from the work by our colleagues showing an increase upon addition of Ca^{2+} in the distance between labels on actin and TnI (Tao et al. 1987).

Studies utilizing proteolytic fragments of TnI (Syska et al. 1976) as well as synthetic peptides (Talbot and Hodges 1981; Nozaki et al. 1980) have identified region 96-116 in TnI (I_{96-116}) as the site of interaction with actin and the segment responsible for the inhibitory properties of TnI. This conclusion has received further support from ^{1}H-NMR work (Grand et al. 1982).

In the present work we have used fluorescently labeled actin or TnI to identify the site of interaction with TnI in the actin sequence. We have found that the crosslinking between actin and TnI with the zero length crosslinker EDC occurs via the N-terminal 12 residue segment of the actin sequence. The same segment has been previously identified as the site of interaction with the heavy chain of myosin subfragment-1 (Sutoh 1982). Addition of myosin S-1 to the actin-TnI complex decreases the yield of crosslinking, without decreasing the binding of TnI to actin as deduced from sedimentation studies, supporting the possibility of multiple interactions between myosin and actin.

MATERIALS AND METHODS

Protein preparations

All proteins were obtained from rabbit back and leg muscles. Actin was prepared according to Drabikowski and Gergely (1962) from aceton dried muscle powder. Troponin was prepared from ether-dried muscle powder and separated into subunits according to Greaser and Gergely (1971). Myosin was purified as described previously (Balint et al. 1975). Chymotryptic myosin fragment S-1 was prepared according to Weeds and Pope (1977).

Labeling of proteins

Troponin I was labeled specifically at Cys-133 utilizing the finding of Chong and Hodges (1982) that only this cysteine is exposed to the solvent in the whole troponin complex. Approximately 50 mg of troponin was dissolved in a solution containing 0.5 M KCl, 50 mM Hepes, pH 7.5, 0.1 mM $CaCl_2$, 6 M urea and 10 mM DTT, followed by dialysis overnight against 0.1 M KCl, 20 mM Hepes, pH 7.5 and 0.1 mM $CaCl_2$. The solution was than incubated for 3 h at room temperature with CPM (2 mol/mol of troponin). The reaction was terminated by addition of 10 mM DTT and the solution was dialyzed against 6 M urea, 25 mM tris-HCl, pH 8.0 1 mM EDTA and 2 mM DTT. The complex was separated into subunits on a Sephadex DEAE A-50 column (1.5 x 20 cm) in a linear gradient of 0-0.5 M KCl. Fractions containing CPM.TnI

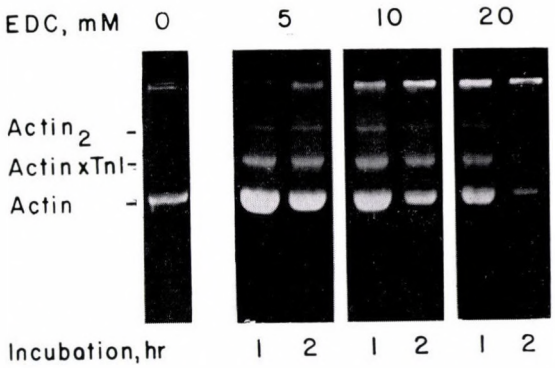

Fig. 1. Crosslinking of CPM. actin and TnI. A 1:1 mixture of CPM.actin with TnI (50 μM) was incubated with EDC for times and at concentrations indicated. Conditions: 50 mM Hepes pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP, 2 mM $MgCl_2$, 0.1 M KCl. Electrophoresis on 10% polyacrylamide gels in the presence of SDS. The gels were photographed in UV light to visualize the bands carrying the fluorescent label

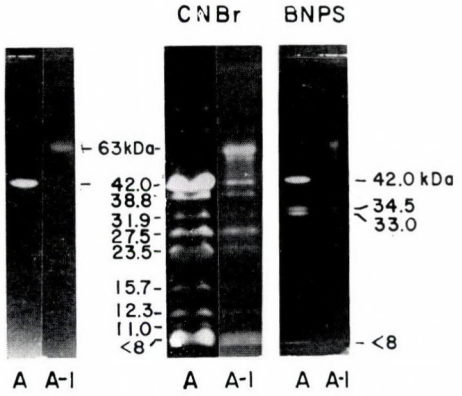

Fig. 2. Digestion with BNPS-skatole (A) and with cyanogen bromide (B). CPM.actin and the crosslinked CPM.actin-TnI complex occluded in cut-out bands following electrophoresis of the EDC treated complex were incubated for 4 h at 45 °C in a solution of BNPS-skatole (1 mg/ml) in 60% acetic acid (A) or for 1 h at room temperature with a 20 mM solution of cyanogen bromide in 70% formic acid (B). Electrophoresis on 15% polyacrylamide gels in a modified Laemmli system (Thomas 1978); the photograph was taken in UV light

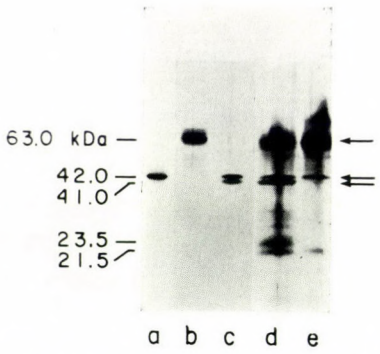

Fig. 3. Digestion with hydroxylamine. CPM.actin and the crosslinked CPM.actin-TnI complex were digested for 3 h at 45 °C with a solution of 2 M hydroxylamine, pH 9.0. Key: a) actin; b) actin-TnI; c) actin incubated with hydroxylamine; d) actin-TnI complex incubated with hydroxylamine; e) actin-TnI complex incubated at pH 9.0 without hydroxylamine. Electrophoresis was carried out on 15% polyacrylamide gel in a modified Laemmli syestm (Thomas 1978) followed by silver staining (Morrissey 1981). Fluorescent bands are indicated by arrows. Note the presence of the 41 kDa band in (c) and (d) and of the 23 kDa band in (d). Small amounts of actin and TnI in (d) and (e) result from nonspecific cleavage of crosslinks at high pH

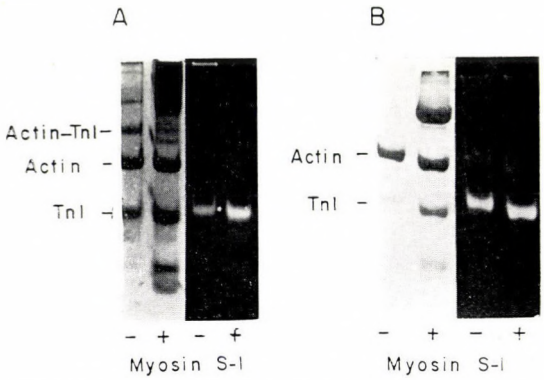

Fig. 4. Effect of S-1 on the actin-TnI interaction. A 1:1 mixture of actin with CPM.TnI was incubated with EDC (A) or sedimented by centrifugation at 100,000× g for 1 h. (B) in the absence (−) or presence (+) of S-1. In each pannel the picture taken under UV light is on the right and that of the gel stained with Coomassie Blue on the left

(identified by polyacrylamide gel electrophoresis) were collected, concentrated and dialyzed against 10 mM Pipes, pH 7.0, 0.5 M KCl. The concentration of the label was estimated from UV absorption at 387 nm using a molar extinction coefficient ε=29,700 $M^{-1}cm^{-1}$ (Sippel 1981); the protein concentration was estimated using the Bio-Rad reagent (Bradford 1976). A typical labeling ratio was 70%.

Actin was labeled at Cys-374 by incubation in the G-form (1-2 mg/ml in 2 mM Hepes, pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP) for 2 h at room temperature with two mol of CPM (added from a concentrated solution in dimethylformamide) per mol of actin. Labeling was terminated by addition of 10 mM DTT and actin was polymerized by addition of 2 mM $MgCl_2$ and 0.1 M KCl. CPM labeled F-actin was separated by ultracentrifugation for 20 min at 220,000 x g in a Beckman TL 100 ultracentrifuge. The pellet was depolymerized in G-buffer (2 mM Hepes, pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP) followed by ultracentrifugation as above to remove insoluble material. The supernatant containing CPM.actin in the G-form was collected and after determination of protein and label concentration 2 mM $MgCl_2$ and 0.1 M KCl were added.

Preparation of the labeled Actin-TnI complex

TnI was combined with F-actin in a 1:1 molar ratio (the CPM label being either on actin or on TnI) in a solution containing 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 M KCl, 50 mM Hepes, pH 7.5 and incubated with 10 mM EDC for 1 h at 25 °C. The crosslinking reaction was terminated by addition of 20 mM DTT, and the sample was dialyzed overnight against a solution containing 1% SDS, 1 mM DTT, 0.125 M Tris/HCl, pH 6.8. After addition of glycerol (final concentration 10% v/v) and bromophenol blue the proteins were separated on 15% polyacrylamide gels in a modified Laemmli system (Laemmli 1970; Thomas 1978). The bands containing actin and the crosslinked actin-TnI complex were identified under UV light, cut out and stored at 0 °C for further studies.

Chemical cleavage of proteins

Proteins trapped in slices of polyacrylamide gel were cleaved with hydroxylamine, BNPS-skatole or CNBr essentially according to the methods described by Sutoh (1982,1983), except that the washing of the gels with increasing concentrations of methanol was omitted and solutions containing proteolytic agent were directly applied to the slices. For digestion at Asn-Gly bonds the gel slices were incubated for 3 h at 45 °C with a 2 M hydroxylamine solution previously adjusted to pH 9.0 with 4.5 M LiOH. Then the gels were washed 3 times for 0.5 h with water and equilibrated with the sample buffer used for SDS polyacrylamide gel electrophoresis. Digestion at tryptophan residues was performed using a solution of BNPS-skatole (1mg/ml) in 60% acetic acid. The gels were incubated for 4 h at 45°C, followed by washing and equilibration with the sample buffer as in the case of hydroxylamine cleavage. For digestion at methionine residues the gels were incubated for 1 h at room temperature with a solution of 20 mM CNBr in 70% formic acid. The reaction was stopped by washing the gels three times with 50% methanol containing 20 mM β-mercaptoethanol followed by equilibration with the sample buffer. As a reference in all cases slices of gels were incubated under similar conditions in the absence of the proteolytic agent. Digested samples were analyzed by electrophoresis on 15% polyacrylamide gel (Laemmli 1970; Thomas 1978). To visualize the bands carrying fluorescent probes the gels were photographed in UV light before staining with coomassie blue. When necessary gels were also stained with silver (Morrissey 1981).

RESULTS AND DISCUSSION

Crosslinking of TnI with actin using the zero-length crosslinker EDC

We have used the zero-length crosslinker EDC, previously succesfully used in studies of myosin-actin interaction (Mornet et al. 1981; Sutoh 1982,1983), to obtain a covalently crosslinked complex between actin and troponin I. The advantage of EDC versus other crosslinkers is that only residues that are in direct contact can be crosslinked which is essential for precise delimitation of the interaction site(s). Although EDC is nonspecific in the sense that any COOH group can be activated, the crosslinking requires a charge-charge interaction of the type COO^--NH_3^+ between the two proteins. Thus the occurrence of crosslinking upon incubation with EDC indicates the presence of such interactions at the interface; however, the lack of crosslinking does not exclude a binding site involving, for example, hydrophobic contacts. The use of EDC for studying interactions in multicomponent systems such as the fully assembled thin filaments is not feasible since each of the components interacts with at least two other components. In these studies we used the binary actin-TnI complex tentatively assuming that if there is a specific site of binding for TnI in the actin sequence the two proteins will also interact at the same site whether or not the other components of the filament are present.

Although under native conditions the ratio of TnI to actin is 1:7 a higher binding ratio of 1:1 can be achieved in the absence of tropomyosin by adding TnI in excess over actin. We have labeled F-actin with a fluorescent probe CPM, at Cys-374, the penultimate residue. CPM.actin was mixed with TnI in a 1:1 molar ratio and incubated with various concentrations of EDC. Fig. 1 shows that the crosslinked actin-TnI complex can be obtained under relatively mild conditions (5 mM EDC, 1 h incubation at room temperature). Increasing the time of incubation or concentration of the crosslinker results in a slight increase in the yield of the crosslinked complex; prolonged incubation with EDC, however, results in increased amounts of crosslinked F-actin polymers rather than in an increased yield of the complex. For the preparation of the actin-TnI complex we have actually used 1 hour incubation with 10 mM EDC.

Identification of the site of crosslinking

Sutoh (1982) has shown that the site or sites of interaction between two proteins can be readily identified using polyacrylamide gel electrophoresis if one of the components is labeled at the N or the C-terminus. The method is based on a comparison of the electrophoretic patterns of fragments containing the label, after limited digestion of the labeled protein alone, and its crosslinked complex with the other component. The use of proteolytic agents of different specificity makes it possible to narrow down the limits of the interaction site. We have used Sutoh's method to identify the site of crosslinking between actin and TnI

Gel slices containing electrophoretically separated CPM.actin and the crosslinked CPM.actin-TnI complex were treated with a number of proteolytic agents. Digestion at tryptophan residues with BNPS-skatole led to formation of three fluorescent bands with apparent masses of 34 kDa, 33 kDa and <8.0 kDa corresponding to residues 75-375, 80-375 and 341(357)-375, respectively, (Fig. 2, Table 1). All three fluorescent bands were present in both the digest of CPM.actin alone and the crosslinked CPM.actin-TnI complex, which indicates that no crosslinking occured between position 75 and the C-terminus. For if any part of TnI had been crosslinked to this portion of actin molecule the position of one or all of the fluorescent peptides in the digest of the complex would be shifted with respect to those resulting from the digestion of free actin. Therefore the crosslinking must have occured somewhere within the N-terminal 74 residue stretch.

To narrow down the limits of the binding site we have digested CPM.actin and the CPM.actin-TnI complex at methionine residues with cyanogen bromide. The patterns of fluorescent bands obtained from the two samples were identical (Fig 2B) and the masses of the fragments were in good agreement with those calculated on the basis of known positions of the methionine residues (Table 1). Even the 39 kDa fragment corresponding to residues 45-375 was present in digests of both the free actin and the actin-TnI complex. Using similar arguments as in case of the digestion with BNPS-skatole we conclude that the site of interaction with TnI is between the N-terminus and Met-44.

Table 1

Fluorescent peptides obtained upon limited proteolysis of CPM.actin by BNPS-skatole and cyanogen bromide.

BNPS-skatole			Cyanogen bromide		
Cleavage point	Mass Calc.	Found	Cleavage point	Mass Calc.	Found
(residue no.)	(kDa)		(residue no.)	(kDa)	
none	42.0	42	none	42.0	42
74	33.7	34	44	37.0	39
85	32.4	33	47	36.8	
339	4.0	<8	82	32.8	32
			119	28.5	28
			123	28.0	
			132	27.0	
			176	22.4	23
			190	20.8	21
			227	16.4	16
			268	12.0	12
			282	10.4	11
			298-354	8.5-2.3	<8

In case of the CPM.actin-TnI complex the pattern of fluorescent bands of mass <42.0 kDa is identical with the corresponding pattern obtained from actin alone. In the regions of the peptides marked by vertical lines only one band was actually found. This may be due to lack of resolution in our electrophoretic system or to negligible cleavage at one or more of the expected sites.

Abbreviations: TnC, calcium binding component of troponin; TnI, inhibitory component of troponin; TnT, tropomyosin binding component of troponin; S-1, myosin subfragment 1; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl] -3-bromoindolenine; CPM, 7-diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethyl-amino)propyl]carbo-diimide; Hepes, 4,(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4 piperazine-diethanesulfonic acid.

Sutoh (1982) has shown that hydroxylamine cleaves actin specifically between Asn-12 and Gly-13. We have followed Sutoh's procedure in an attempt to further delimit the binding site. Cleavage of CPM.actin with hydroxylamine yielded a 41 kDa fluorescent fragment corresponding to residues 13-375 of actin (Fig. 3, Table 2). The same fluorescent fragment was obtained from the CPM.actin-TnI complex. This result suggests that the site of crosslinking to TnI is within the N-terminal 12 residue stretch of actin.

There is also a nonfluorescent 23 kDa peptide in the digest of the complex but not in the digest of free actin. In order to identify the 23 kDa peptide we have labeled TnI with a fluorescent probe (CPM) at Cys-133 - the only cysteine residue in the troponin complex accessible to the solvent (Chong and Hodges 1982). Hydroxylamine digestion of the CPM.TnI-actin complex produced the same pattern of cleavage as that of the CPM.actin-TnI complex, except that now the 23 kDa peptide was fluorescent, supporting the idea that it represents TnI crosslinked with the 12 residue N-terminal fragment of actin (Table 2).

These results unequivocally identify the N-terminal twelve-residue segment in actin as a site of interaction with TnI.

Effect of myosin S-1 on TnI-actin interaction

Recently Sutoh (1982) has reported that the heavy chain of myosin subfragment 1 binds to the N-terminal 12-residue segment in actin. Our present findings raise the possibility of competition between TnI and S-1 for a common binding site on actin. To check this point we have investigated the binding to thin filaments of TnI labeled with CPM at Cys-133 in the presence and absence of S-1. Although in the presence of S-1 the level of the crosslinking between actin and TnI was decreased (Fig. 4A), there was no decrease in the binding of TnI to actin as indicated by the level of fluorescence in the pellets upon high speed centrifugation (Fig. 4B). Recently Miller et al. (1987) have shown that an antibody specific for the N-terminal seven residue segment in actin does not interfere with S-1 binding and cosediments with actin and S-1 on high speed centrifugation. Both their results and ours may be readily explained by the existence of a second

Table 2

Identification of bands resulting from cleavage of actin and the actin-TnI complexes with hydroxylamine.

Sample	Mass			Identification
	Apparent		Expected	
	Fluor.	Nonfluor.		
		(kDa)		
CPM.Actin	42		42.0	CPM.Actin
	41		40.5	CPM.Actin$_{13-375}$*
CPM.Actin-TnI	62		63.0	CPM.Actin-TnI
	42		-	CPM.Actin**
	41		40.5	CPM.Actin$_{13-375}$
		23	22.3	TnI-Actin$_{1-12}$
		22	-	TnI**
CPM.TnI-Actin	61		63.0	CPM.TnI-Actin
		42	-	Actin**
		41	40.5	Actin$_{13-375}$
	23		22.3	CPM.TnI-Actin$_{1-12}$
	21		-	CPM.TnI**

*) Subscripts refer to numbering of residues in the amino acid sequence.

**) The presence of small amounts of undigested individual components of the complex results from unspecific cleavage of the crosslink upon incubation at high pH. These bands are also formed when no hydroxylamine is present.

binding site between actin and S1 (Mornet et al. 1981; Sutoh 1982; Trayer et al. 1987) permitting the binding between actin and myosin even if the interaction at the N-terminus is blocked by TnI or the antibody. This would also indicate that the interaction between myosin and the N-terminus of actin is relatively weak. The importance of this interaction, if any, remains to be evaluated.

CONCLUSIONS

The N-terminal segment of actin contains a cluster of negatively charged residues (Asp-Glu-Asp-Glu) which have been implicated in the interaction with several actin binding proteins including myosin (Sutoh 1982), fragmin (Sutoh 1986), depactin (Sutoh 1984) and plasma-gelsolin (Doi et al. 1987). The present studies add the inhibitory component of troponin to this list. Previously a segment of TnI containing residues 96-116 (I_{96-116}) has been identified as the site of interaction with actin and the region responsible for the inhibitory properties of TnI (Syska et al. 1976; Grand et al. 1982; Talbot and Hodges 1981; Nozaki et al. 1980). Since I_{96-116} contains a number of lysine and arginine residues our present study suggests that the interaction between TnI and actin and possibly the inhibition of actomyosin ATPase involve a charge-charge type interaction between segment I_{96-116} and the segment 1-12 of actin (A_{1-12}). It should be recalled that the inhibitory fragment of TnI (I_{96-116}) interacts not only with actin but also with the stretch of residues 89-100 of troponin C (C_{89-100}) which has been found indispensable for the Ca^{2+}-dependent regulatory properties of troponin (Grabarek et al 1981). We have also shown that Ca^{2+}-binding to the N-terminal low affinity sites in TnC induces fast conformational changes in region C_{89-100} (Grabarek et al 1986). Thus it is very likely that the Ca^{2+}-dependent regulation in skeletal muscle involves alternation of region I_{96-116} between actin site A_{1-12} in the absence of Ca^{2+} and the TnC site C_{89-100} at micromolar Ca^{2+} concentrations.

We realize that the above model, although attractive, does not take into account tropomyosin. Since in native thin filaments six out of seven actins are in contact with tropomyosin rather than with TnI one may ask what the relation between the TnI-actin interaction and the tropomyosin-actin interaction is. Does the inhibition of actomyosin ATPase by tropomyosin

involve the same site on actin as TnI does, or is the inhibition involving tropomyosin based on a different mechanism than that brought about by TnI alone? These questions will be addressed in future studies.

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FROM MYOSIN "CRYSTALLIZATION" TO FILAMENTOGENESIS*

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Only through constant reminder and reiteration will the tortuous continuum of human progress be remembered - and we dare not forget it if we are to move forward and enrich the future. So I will recall the days when under severe hardships during the war period and the political turbulence of an impending invasion of Hungary by Nazi Germany, Albert Szent-Gyorgyi ran a most exciting and extremely productive biochemistry laboratory in Szeged. As is well known, Szent-Gyorgyi and his associates particularly Banga and Straub, made a number of fundamental discoveries during the years 1940-1944, laying the foundation of our current understanding of the molecular mechanisms of muscle contraction. The discoveries were briefly and precisely written up and a few hundred copies of 3 volumes of "Studies from the Institute of Medical Chemistry, University of Szeged" (1) were printed by the local printing shop. To ensure against the loss of the results and conclusions, if he should be killed by the Nazis, Szent-Gyorgyi managed to send the Szeged series of articles (in modified form) to his friend Hugo Theorell in Stockholm for publication in Acta Physiologica Scandinavica (2). Apparently, this correspondence with Theorell, enabled the Nazis to track down Szent-Gyorgyi at the Swedish Embassy in Budapest, where he was given refuge, but he managed to escape in the nick of time.

* *In memoriam Albert Szent-Györgyi*

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This and other incredible adventures during this perilous period are described by Szent-Gyorgyi in an autobiographical sketch "Lost in the Twentieth Century" (3).

The fundamental discoveries made in Szeged were on myosin, as it was known at the time. Banga and Szent-Gyorgyi found (1,2) that it could be extracted in two forms depending on the length of the extraction period. The protein after 24 hrs extraction, named myosin B, was far more viscous than myosin A obtained after 20 min of extraction; the viscosity of myosin B could be reduced by ATP. The next important milestone was Szent-Gyorgyi's production of shortening of a thread of myosin B by exposing it to a water extract of muscle. Through incisive deduction and simple experiments he determined that the shortening of the myosin thread was caused by ATP in the presence of Mg and K. To see the contraction of these threads for the first time was according to Szent-Gyorgyi the most thrilling moment of his life. Straub, obviously impressed, wrote "To be privileged to have a look at it, as Albert Szent-Gyorgyi proudly demonstrated it to us in the laboratory in 1940 was a great thing" (4). Myosin A did not shorten on exposure to ATP and salts; myosin B apparently contained another protein (missing in significant amounts in myosin A) designated actin, which was elegantly purified and characterized by Straub (1, 2). After Szent-Gyorgyi purified and "crystallized" myosin (to be discussed later), the two contractile proteins, myosin and actin (actomyosin) were clearly shown to interact with ATP resulting in shortening of an actomyosin thread or in superprecipitation associated with hydrolysis of ATP (1,2). Szent-Gyorgyi with remarkable insight concluded that this interaction was the fundamental process of contraction in living muscle. This idea, however, was not readily accepted when it became more widely known after the publication of his booklet on Chemistry of Muscle

Contraction (5). On reviewing the book, Bailey (6) considered it as a "rather novel and daring approach to the problem of physiological contraction". Amongst various criticisms he wrote, "In all that concerns the properties of proteins there are serious misconceptions." "...to transpose this system [actin-myosin-ATP] to muscle itself is not to offer an explanation of how muscle contracts." "...it [the book] should not be recommended to uncritical and over-receptive students." Fortunately, there were those who were very receptive and excited by the book which opened the frontier of muscle research.

By that time Szent-Gyorgyi had left Hungary painfully disenchanted with the ruthless domination of Hungary by the Soviet Union and he established the Institute for Muscle Reserch at the Marine Biological Laboratory, Woods Hole, USA and there he finally overcame the criticisms of his basic theory of muscle contraction. Whereas actomyosin threads shortened but could not produce tension, Szent-Gyorgyi's glycerol extracted psoas muscle preparation (which could be preserved for long periods of time in the freezer at -20°C) contracted in the presence of ATP generating tension comparable to that of living muscle (7). In this preparation, free of many soluble substances, the structural organization of the contractile elements is preserved. Hugh Huxley regards the glycerol extracted muscle preparation as "undoubtedly the most important and far reaching innovation introduced by Szent-Gyorgyi". He continues: "This type of preparation is of inestimable value, as everyone who works on muscle will agree and its usefulness was appreciated and exploited rapidly" (8).

The specific Szeged contribution particularly relevant to the subject of this article is Szent-Gyorgyi's "crystallization" of myosin (1,2). After several steps of muscle extraction Szent-Gyorgyi produced very fine "needles", seen under the light microscope, by diluting the KCl in which

the myosin was dissolved to a concentration of 0.04 M. These so called crystals were later determined electron microscopically to be aggregates of myosin filaments (9, 10). Szent-Gyorgyi found "that crystallization is limited to a narrow pH range and has its maximum at about pH6-5." He found that myosin in 0.1-0.2M KCl showed a very strong DRF (Double Refringence of Flow). He wrote: "The DRF is not due to the single myosin particles but to their coaxial association, for the DRF becomes weaker at 0.3 MKCl and disappears entirely at 0.4M KCl. It also disappears if the solution is rendered alkaline. Evidently the high salt concentration or pH prevents association". As I shall describe, we now know through the use of the electron microscope and ultracentrifuge that pH and salt concentration determine the assembly and size of synthetic myosin filaments.
Szent-Gyorgyi also apparently produced small aggregates at very low ionic strength by dialysis of the crystalline muscle against water. He describes a progressive development of "a glassy mass which shows only a slight opalescence" which then becomes clear and viscous. Bivalent cations ($CaCl_2$ & $MgCl_2$) giving a rather voluminous precipitate is another one of Szent-Gyorgyi's findings (1,2). By studying the migration of myosin coated charcoal particles in a microscopic cataphoretic apparatus, Koloman Laki, at Szent-Gyorgyi's request, determined that myosin dissolved in distilled water is negatively charged and that the myosin is neutralized in the presence of 0.05M KCl when precipitaton occurs. Szent-Gyorgyi also found that ATP disaggregates associated myosin. "All the observations [he concludes] are in agreeement with the assumption that myosin consists of moderately elongated particles which have a strong tendency of lateral association to rather long particles or swarms, the forces holding these swarms together being rather weak." (2).

Thus Szent-Gyorgyi through simple carefully thought out experiments and

remarkable intuitive insights and incisive reasoning established the conditions for the formation of various types of synthetic myosin filaments and their aggregates. Lacking, of course, was any concept by Szent-Gyorgyi of directionality or polarity in the self-assembly of the myosin molecules. This was established by Huxley in 1963 (11) on observing essentially Szent-Gyorgyi's crystalline myosin negatively stained in the electron microscope. At pH 7.0, dilution of the KCl of a myosin solution to 0.2 to 0.1M produced filaments of about 0.5 μm in length if the dilution was rapid, and longer ones, about 1 or 2μm if the dilution was produced by dialysis. The filaments were spindle shaped with projections along their sides. A very significant characteristic of shorter filaments (0.25 to 0.3 μm) was the presence of a bare central zone, 0.15 to 0.20 μm in length with irregular projections at either end. Longer filaments only occasionally showed a similar bare central zone of about the same length. These findings led to Huxley's proposed model of antiparallel stacking of the myosin molecule with partial overlap in the center and continued parallel stacking with reversed polarity on either side of the central zone, leading to a filament of appropriate length. The backbone of the filament is formed by association of light meromyosin (LMM) and the projections at either side of the bare central zone by the heavy meromyosin (HMM) portions of the molecule. This model, universally accepted, conforms with the native filament organization and sliding function in the myofibril. Huxley in describing his findings selected various sized filaments to deduce his model. How certain conditions might influence synthetic myosin filamentogenesis was not addressed. I subsequently undertook such a study, but as is often the case, my interest in the problem actually arose from an accidental observation which I made in Szent-Gyorgyi's laboratory.

I had the privilege and pleasure of joining the laboratory of this

uniquely wonderful man in 1959. In South Africa I was profoundly influenced by his exciting little books on muscle (5,12,13) and became especially fascinated by his penetrating questions and speculations on the possible role of water, the "mater of life," in muscle contraction. Heavy water with properties different from normal water should, I thought affect the contractile process and could be a useful tool in dissecting out some mechanisms in muscle contraction. After pursuing studies on the effects of D_2O on a number of muscle preparations a Rockefeller Fellowship enabled me to join Szent-Gyorgyi's laboratory. I had delayed publication hoping for help from the master of muscle in interpreting some of the results. At my first most memorable meeting with him in Woods Hole he listened very attentively to my description of the findings and interpretations and I eagerly and with trepidation awaited his comments. Szent-Gyorgyi simply pronounced: "The more I know about muscle the less I understand it" (that naturally put me at ease) and he suggested I write up my interesting results. And then excitedly, with his bright blue eyes sparkling, he proceeded to explain simply his current ideas on charge transfer reactions: "A new dimension must be explored to understand the meaning of life." Little did I realize, although I had read his little book Bioenergetics (14), that he had divorced himself from research on muscle for a considerable period of time. My knowledge of charge transfer complex reactions was superficial, so with a mixture of concern and excitement I accepted the challenge. Fortunately, Irvin Isenberg, a biophysicist in the laboratory, who became my closest friend, lent me his shoulder to lean on. Gently and kindly "Prof" as Szent-Gyorgyi was endearingly called, suggested that I try out his ideas on an invertebrate muscle readily available at the Marine Biological Laboratory. Prof himself with remarkable powers of concentration daily devised experiments and formed new charge-transfer

complexes in test tubes. As he observed the color changes and formulated his ideas he imparted an exhilarating excitement and lust for discovery I tried numerous experiments using electron donors and acceptors on a number of preparations. One observation I made was on glycerinated muscle, which to my surprise, stained metachromatically with toluidine blue. For one fleeting moment Szent-Gyorgyi was recaptured into muscle research. He used toluidine blue as a marker to identify a protein which we extracted from muscle and he designated it "metin". We pointed out that it resembled tropomyosin and also reported on the presence of a minor component in the preparation detected by ultracentrifugation (15). These findings were made prior to Ebashi's publication on "native tropomyosin" (16). Subsequently Azuma and Watanabe (17) showed that our minor component was one of Ebashi's proteins in native tropomyosin later to be identified as troponin (18). At the time when there was no known function ascribed to tropomyosin I attempted a systematic study on the effect of the metin preparation on superprecipitation of actomyosin at different pH's and temperatures.

A preparation of myosin at pH 8.0 (0.025 M Tris) in 0.08 M KCl went into "solution" showing a marked Tyndall effect. I was intrigued as to the state of the myosin in this colloidal solution and on examining it in the electron microscope found short fairly uniform synthetic filaments varying in length from 0.2 to 0.5 μm (mean: 0.4μm and 60 to 120 Å in diameter). They had bare central shafts and some had large bulbous projections at the ends; these projections were however not prominent in the shorter and thinner filaments within the population. On ultracentrifugation (using a protein concentration of 2.4 mg/ml) there was a hypersharp peak sedimenting at 21S and a minor peak at 6.8S. This preparation, very stable, with a dynamic equilibrium between the two states of myosin, was described by Kaminer and Bell in 1966 (19). Similarly Reisler et. al. in 1980 (20)

illustrated sedimentation coefficients of 20.4S-21.8S (using a protein concentration of 1.7 mg/ml) and a trace amount of monomer in their preparation of myosin in 10mM citrate/Tris buffer at pH 8.0. Electron microscopically the filaments had a mean length of 0.303 μm (87% ranging narrowly between 0.275 to 0.325 μm) with a mean diameter of 82 Å resembling the smaller filaments illustrated by Kaminer and Bell (19,21). The authors designated their preparation myosin minifilaments contrasting them in their publication and in subsequent ones, with synthetic myosin filaments. Etymologically, such terminology for comparison, I consider in passing, is inappropriate and could be confusing since minifilaments are simply a particular form of synthetic myosin filaments of which a variety have been produced in terms of size and other characteristics depending on the conditions of preparation (11,21,22,23). By adding various concentrations of KCl Reisler et. al. in 1982 (24) produced a certain amount of growth of the filaments in the presence of 70mM KCl leading to a mixture of synthetic mini- and somewhat longer filaments varying from 0.25 to 0.6 μm. This length distribution is closer to that in our preparation (19) in the presence of 80 mM KCl which, in retrospect, contains a mixture of minifilaments and somewhat larger ones. In the presence of higher concentrations of KCl (0.1M) all the minifilaments in Reisler's preparation apparently increase in length; this growth is ascribed to an initial dissociation of monomers with subsequent reassociation into longer filaments (24). Reference to these findings will be made again later.

Following on this initial study we investigated the myosin self-assembly process and filamentogenesis(21). By rapid dilution of the KCl concentration (0.6 M KCl) to 0.3, 0.2 and 0.1 M we could observe the progressive formation of longer filaments. The important effect of pH on filamentogenesis was simultaneously determined by carrying out the

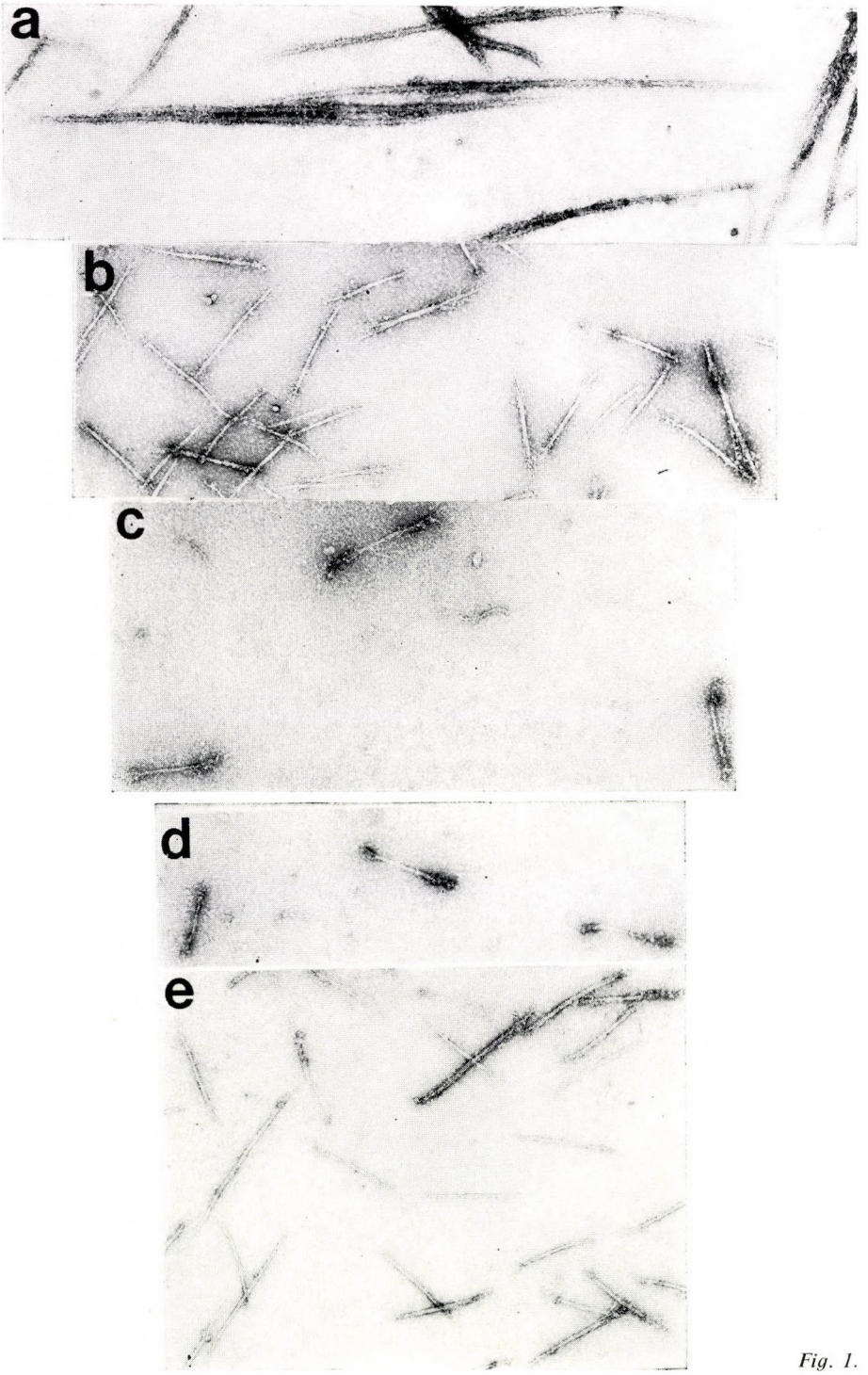

Fig. 1.

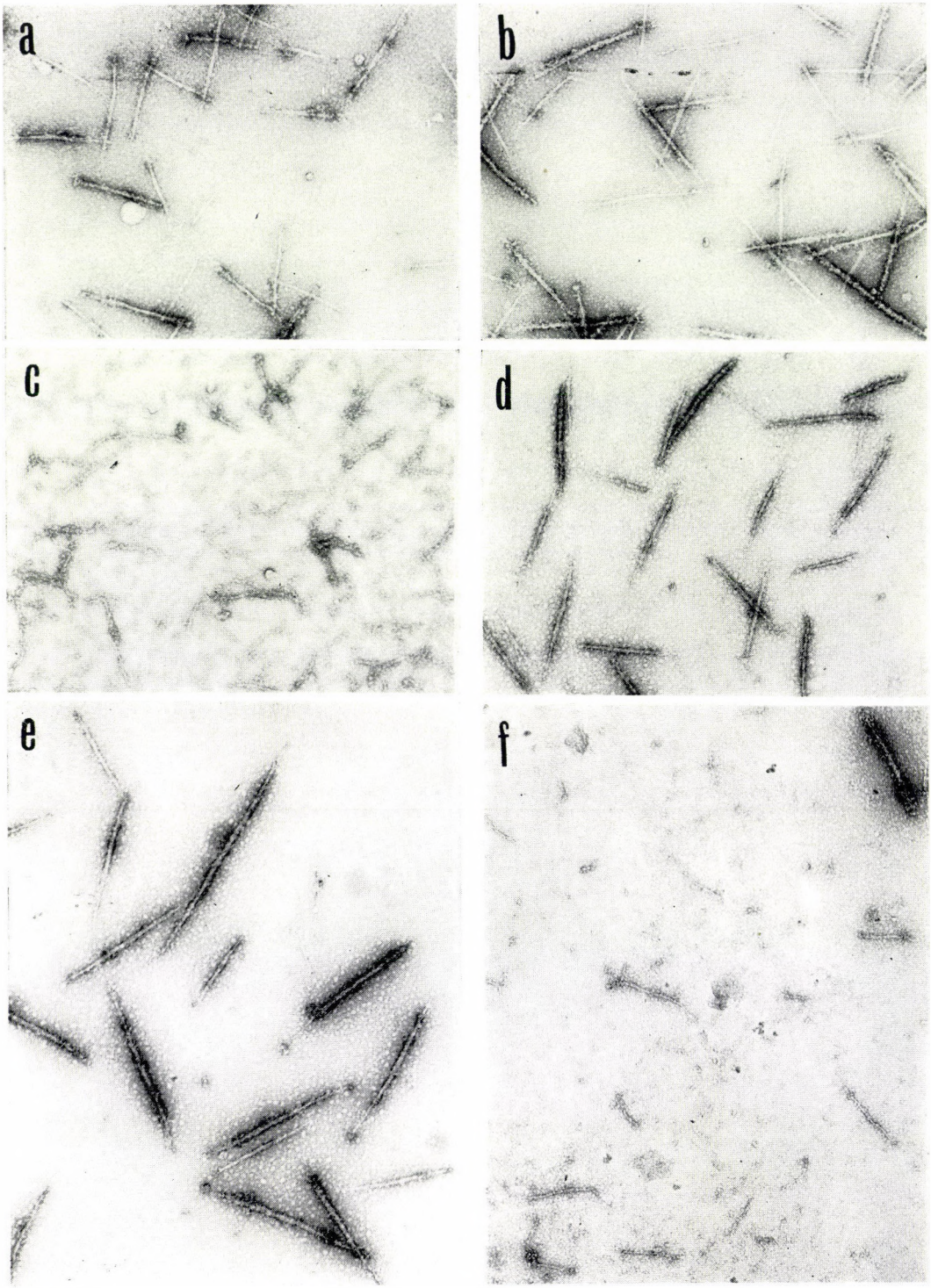

Fig. 2.

Fig. 1. Synthetic myosin filaments negatively stained with uranyl acetate. × 26,900. (a) Long tapered filaments formed by rapid dilution to 0.1 M KCl pH 6.5. (b) Same preparation as (a) 1/2 hour after increasing the pH to 8.0. The filaments have become shorter and thinner many lost their projection along the shaft and developed a bare central zone. Some fraying occurs at the ends which lose their tapered appearance. (c) Same preparation as (b) 2 hrs after increasing the pH to 8.0. The continued dissociation of monomers results in the typical bipolar filaments with a bare central shaft and bulbous projections at the ends. (d) A different preparation of filaments formed by rapid dilution to 0.1 M KCl at pH 8.0. (e) Same preparation as (d) after reducing the pH to 6.5. The filaments have grown in size (to a limited degree as described in text), projections appear along the shaft and the bulbous projections become less prominent at the ends which tend to become tapered

Fig. 2. Synthetic myosin filaments stained as in Fig. 1. × 26,900 (a) Filaments formed by rapid dilution of KCl to 0.1 M KCl at pH 7.5. Filaments are somewhat longer than those at pH 8.0 (Fig. 1d), and with more projections along the shaft. (b) Same preparation as (a) after decreasing the pH to 6.5. There is some growth of the filaments and tapering at the ends. (c) Filaments formed by rapid dilution of KCl to 0.2 M KCl at pH 7.5. Short twig-like filaments. (d) Same preparation as (c) after decreasing the pH to 6.5. The twig-like filaments have grown in length forming spindle shaped filaments. (e) Filaments formed by rapid dilution to 0.2 M KCl at pH 6.5. The filaments are spindle shaped. (f) Same preparation as (e) after increasing pH to 7.5. Myosin dissociated off the spindle shaped filaments forming short twig-like filaments

experiments at different pH values (8.0, 7.5, 7.0, 6.5 and 6.0). Thus the state of aggregation of the myosin was observed by us at each defined stage of KCl dilution at a particular pH. Each preparation was examined in the electron microscope by negative staining (11) and in the analytical ultracentrifuge. Using flow birefrigient techniques quantitatively, Noda and Ebashi (25) in 1960 had already demonstrated the effects of pH and ionic strength on the aggregation of myosin; polymers of 1 μm were formed at low ionic concentration at pH 6.2.

We demonstrated the assembly of myosin into progressively larger filaments as the KCl concentration is diluted stepwise from 0.6M to 0.1M with the longest ones occurring at pH 7.0 to 6.5. Short twig-like filaments (0.22μm x 40-80Å) are formed in 0.3M KCl at pH 7.5 - 6.5 and somewhat longer ones (0.4μm) at pH 6.0. Further growth occurs as dilution proceeds to 0.2M KCl (pH 8.0-7.0). So far a certain amount of monomer remains dissociated in the above preparations but at pH 6.5-6.0, with further growth, monomer is no longer detected. The filaments, now spindle shaped, are about 1.1μm x 180-300Å at pH 6.5 and somewhat shorter at pH 6.0.

On further dilution to 0.1M KCl larger spindle shaped filaments resembling more closely the natural thick ones are formed (av: 1.0μm at 7.0, 1.8μm at 6.5 and 0.96μm at pH 6.0). At pH 8.0, characteristic bipolar filaments are formed, 0.22 to 0.67 μm (av: 0.43 μm in length x 60-120 Å). They have bare central shafts (0.15 to 0.25 μm) and many show bulbous grossly irregular projections at the ends; the smaller ones have less prominent projections. At pH 7.5 these filaments are somewhat longer with less bulbous projections. A few of these bipolar filaments are also seen at 0.2M KCl, pH 7.0 resembling the ones selected by Huxley (11) for his proposed model on the assembly process. These distinctly bipolar types of

filaments are of course similar to the ones we previously described at pH 8.0 in .08 M KCl (19). But this preparation in 0.1M KCl has a larger proportion of the longer filaments with prominent projections at the ends and on ultracentrifugation two aggregate peaks, 34S and 77S and a monor (6.9S) are seen (conc. of protein - 0.6 mg/ml). Reisler et. al. in 1982 (24) likewise obtained two aggregate peaks of 28.2S and 42S and a monomer peak of 6.1S by adding 0.08 M KCl to the minifilaments by dialysis and a single 46S aggregate and a monomer peak in the presence of 0.1 M KCl (conc. - protein was 3 mg/ml). The minor peak of 6.9S in our preparation is probably indicative of a mixture of monomer and dimer (26) to be discussed later. Overall there is a correlation between the diameter and lengths of the filaments; as the filaments grow in length there is usually also an increase in diameter.

We also induced either dissociation or growth of preformed filaments at a particular KCl concentration, by altering the pH in one or other direction. Illustrating these results are Figs. 1 and 2 which were omitted from the article (21). By increasing the pH of 6.5 (at 0.1 M KCl) of a preparation to 8.0 the long tapered filaments dissociate fairly rapidly changing progressively to typical bipolar filaments with bare central zones (Fig 1 a,b,c). They obviously shorten symmetrically on either end towards the center and the dissociated monomers are detected in the ultracentrifuge. Conversely, on decreasing the pH of 8.0 to 6.5 (at 0.1 M KCl) the length of the filaments increases (Fig. 1 d,e) to a degree limited by the available monomers which are no longer detectible. Growth obviously occurs at both ends which become less bulbous and more tapered due presumably to swinging of the cross bridges towards the core of the filament. Interestingly, the bare central zones in some filaments disappear due presumably to lateral imperfect stacking of monomers or

dimers in the center. We had suggested that as more molecules stack forming longer filaments there is a greater chance for errors and imperfection accounting for the rarity of bare central zones in long synthetic filaments; on dissociation of monomers there is reappearance of the bare zone as is evident in figure 1 a,b,c.

Similarly decreasing the pH of 7.5 (in 0. M KCl) to 6.5 results in some increase in length and tapering of filaments (Fig. 2a,b). Twig like filaments at pH 7.5 (0.2 M KCl) double in length and become tapered on altering the pH to 6.5 (Fig. 2c,d) and monomers disappear from the preparation. Conversely spindle filaments dissociate into short twig like ones on increasing the pH of 6.5 (in 0.2 M KCl) to 7.5 (Fig. 2e,f) and dissociated monomers are detectable. Occcasional bipolar filaments are seen, presumably an intermediate stage in the dissociation into twig-like filaments.

Similar types of experiments have since been carried out on native thick filaments. Niederman & Peters in 1982 (27) dissociated native filaments by dialysis against a solution at pH 8.0 (0.1 M KCl) or a solution of 0.2 M KCl (at pH 7.0). The normal filaments dissociate into typical bipolar ones (called bare zone assemblages) which re-assemble on reconstituting the pH to 7.0 or the KCl to 0.1 M. Stepwise increases in KCl conc. (up to 300 mM) cause dissociation of native thick filaments producing stubs (28) of varying lengths some of which (at 190 mM KCl) resemble the typical bipolar filaments with bulbous projections.

A detailed and careful study of the sedimentation coefficients of synthetic myosin filaments was reported in 1966 by Josephs & Harrington (22) correlating them with the size of the filaments determined electron microscopically. Filaments produced by dialysis against solutions with various pH's and KCl concentrations were examined. Between pH's of 6.22 and

7.1 a number of classes of polymers were described. The lengths of filaments in one class vary from 2-12 μm with sedimentation coefficients of 1100S and 3000S often containing a monomer. In another there is a narrower length distribution of 0.4-3.0μm (330S and 180S). In the pH range of 8.0-9.5 shorter filaments are found (70% between 0.56 and 0.75μm (av: 0.63μm)). At infinite dilution the sedimentation coefficent is 150S and a monomer is always present. These filaments were studied in more detail and the approximate equilibrium constant between monomer and polymer was determined.

In the assembly process myosin dimers may be involved; they are present in solution at high ionic strength (29). Katsura and Noda (30,31) and Herbert and Carlson (32) reached similar conclusions. The presence of dimer was later demonstrated electron microscopically by Davis et. al. in 1982 (33). Another approach in studying myosin filament assembly arose out of a finding by Josephs and Harrington in 1967 (34) who caused dissociation of myosin polymers in the ultracentrifuge by increasing the hydrostatic pressure. Davis in 1981 (35,36), using a pressure jump apparatus partially dissociated synthetic myosin filaments formed in 0.15 M KCl, pH 8.1 and then reassembled them by rapid return to atmospheric pressure. From turbidity measurements he concluded that dissociated monomers first form parallel dimers which then add to the partially dissociated filaments at a fixed rate. The rate of dissociation of filaments on the other hand is length dependent increasing as the filaments grow to a point where an equilibrium is established between association and dissociation resulting in cessation of growth; these types of studies formed the basis of a proposed model on length regulation by Davis (37).

A reasonable notion is that filamentogenesis and also the length of filaments depend on the proper array of complementary positive and negative

charges and hydrophobic regions on the myosin molecule available for self-assembly. The H^+ and ionic concentration in the environment will naturally influence the ionic charges of the complementary site in the myosin molecule. Rapid dilution of the KCl, one can imagine, will first form relatively large numbers of myosin nuclei, onto which the remaining monomers, relatively few in number, will associate thus forming shorter and more uniform filaments compared to those formed by slow dilution of the KCl (11, 30, 38, 39). Upon slow dilution fewer nuclei and consequently longer filaments will form, particularly during slow stirring of a dialysis bag. The resultant slow and uneven mixing could produce a gradient of KCl concentrations from the center to the periphery of the dialysis bag at an early stage of dialysis. The relatively few nuclei formed at the periphery of the bag, where the KCl would first be reduced, could then attract abundant numbers of available monomers to form long filaments with a wide distribution of lengths.

The presence of divalent ions also influences the assembly of myosin as Kaminer et al reported in 1973 (40). In the presence of $CaCl_2$ or $MgCl_2$ shorter filaments are formed (Fig. 3). The precipitation appeared to form more rapidly than in the samples without divalent cations suggestive of more rapid filament formation due to a positive aggregative influence by the divalent cations. Pinset-Harstrom and Truffy in 1979 (41) found no effect of $Ca\ Cl_2$ and $Mg\ Cl_2$ in the formation of long filaments (5-15μm) by very slow dilution. However the divalent ions prevented ATP-induced dissociation of filaments. Pinset-Harstrom in 1985 (42) showed that excess $Mg\ Cl_2$ in the presence of Mg ATP resulted in thicker and shorter spindle shaped filaments.

Whereas general principles of self-assembly of macromolecules are reasonably well understood (43) the precise details of the myosin assembly

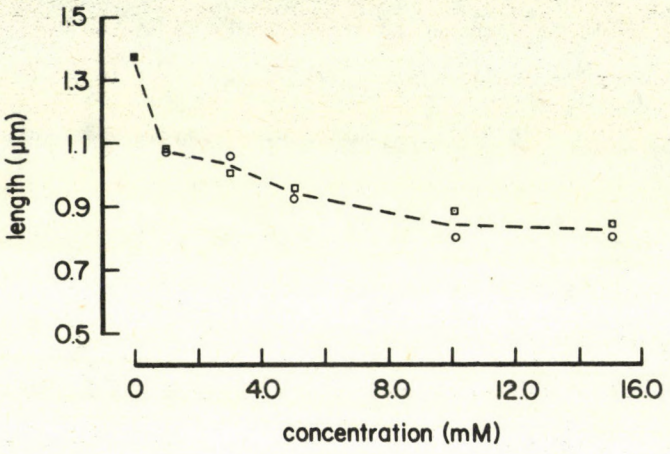

Fig. 3. Average length of myosin filaments at varying concentrations of either $MgCl_2$ or $CaCl_2$. Filaments formed by rapid dilution of myosin in 0.6 M KCl at pH 6.5 (25mM Tris-Maleate), containing varying concentrations of either $MgCl_2$ or $CaCl_2$. The diluting buffer (25mM Tris-Maleate pH 6.5) contained corresponding concentrations of either $MgCl_2$ or $CaCl_2$. The KCl was diluted to 0.1 M KCl. ■ No divalent ions - av. length 1.356μm (S.E. 0.016, n=270). □ Plus $MgCl_2$ 1mM - av. length 1.078μm (S.E. 0.016,n=204). 3mM - av. length 1.001μm (S.E. 0.015, n=217). 5mM -av. length 0.950μm (S.E. 0.015, n=243). 10mM - av. length 0.881μm (S.E. 0.015, n=324). 15mM - av. length 0.841μm (S.E. 0.012, n=225). ○ Plus $CaCl_2$: 1mM - av., length 1.088μm (S.E. 0.014, n=224). 3mM -av., length 1.061μm (S.E. 0.161, n=176). 5mM -av., length 0.926μm (S.E. 0.009, n=279). 10mM - av., length 0.798μm (S.E. 0.114, n=181). 15mM -av., length 0.807μm (S.E. 0.013, n=225).

process are presently beyond reach. Some progress is being made since the complete amino acid sequence of the myosin rod from the worm Caenorrhabditis elegans was deduced. Using this information, McLachlan and Karn in 1982 (44) have schematically drawn a simplified bipolar model of a myosin filament. The surface of the molecule has alternating clusters of positive and negative charges. By appropriate staggering the authors obtain a bare central zone and a proper spacing of myosin cross-bridges with an axial spacing of 143 Å and a helix repeat of 430 Å known to exist in thick filaments (45). Koretz (46) found similar repeats in synthetic myosin filaments. Since the thick filament is known to contain other proteins such as C protein located at 430 Å intervals on either side of the bare zone (47,48), synthetic myosin filament formation was studied in the presence of C protein. It had no influence on the length of long filaments formed by dialysis but it caused some disorganization of the filaments (49,50). On the other hand, Koretz found that C protein, under appropriate conditions stabilized short filaments (51). This interaction of myosin with another protein could support the proposal of Huxley and Brown (45) of a Vernier mechanism for length determination.

The assembly of myosin in a helical fashion forms a number of subfilaments. There is dispute on the number. Pepe and Drucker (52) and Stewart et al (53) provide evidence favoring 12 subunits, whereas Squire (54, 55) presents a 3 stranded model. In support of 3 subfilaments is the interesting finding of Maw and Rowe (56) who showed fraying of natural thick filaments into 3 subfilaments on either side of the central portion on exposure to very low ionic strength.

Synthetic myosin filaments formed from smooth muscle myosin, first observed by Hanson and Lowey in 1964 (57) also showed a dependence on pH and ionic concentration as reported by Kaminer in 1969 (58). The

filamentogenesis is similar to that observed in striated muscle myosin (21) except that the filaments are significantly shorter, the longest at pH 6.5 in 0.1 MKCl, averaging about 0.6μm. Later studies on smooth muscle myosin revealed a number of interesting findings. Onishi and Wakabayashi in 1982 (59) and Trybus et al in 1982 (60) found that monomer, produced by MgATP induced dissociation of smooth muscle myosin filaments, became folded on itself forming a hair-pin loop of the rod. Such folding occurred if the 20,000 MW light chain was dephosphorylated. Dephosphorylated myosin filaments are disassociated by stochiometric amounts of MgATP whereas phosporylated myosin remains mainly assembled. Hence phosphorylation may play a role in the assembly of smooth muscle myosin.

Craig and Megerman in 1977 (61) described the formation of side-polar (rather than bipolar) filaments under certain conditions with cross-bridges of one polarity on one side and opposite polarity on the other side of the filament. Somewhat similar observations were made by Hinssen et al in 1978 (38) who also showed that considerably longer smooth muscle myosin filaments could be formed by slow dialysis. These long filaments resemble natural thick ones isolated from vertebrate smooth muscle by Small in 1977 (62).

Interestingly, Kaminer et al (63), Pollard (64) and Wachsberger and Pepe (65) showed copolymerization of skeletal and smooth muscle myosin into filaments which in 0.1 M KCl at pH 7.0 or 6.5 are shorter than those produced from skeletal muscle myosin. Platelet myosin similarly copolymerizes with skeletal muscle myosin (64). It appears therefore that myosin forming short filaments, under these conditions, limits the length of the "hybrid" filaments.

In concluding this review with a brief reference to myosin filaments in non-muscle cells, I draw attention to the long forgotten experiments of

Albert Szent-Gyorgyi when he extracted, over 45 years ago, contractile proteins from non-muscle cells. In an article "On protoplasmic structure and function" published in 1940 (66), Szent-Gyorgyi refers to his profound conviction that basic processes of life will be the same in all cells and organs. After describing his views on muscle contraction and on myosin (that is myosin B, before actin was identified) he writes "a protein fraction analogous to myosin should be found in any cell." He then proceeds to describe the extraction of a protein from kidney, named renosine, which behaves like myosin. He made similar extracts from liver, brain, nerves, mammary gland, parotid, lymph gland, whole embryos, Rous sarcomas and Ehrlich carcinoma. He also correctly stressed the importance of this cellular material being built of rod shaped molecules. Szent-Gyorgyi, in advance of his time, thus anticipated the discoveries of myosin and actin in non-muscle cells. Myosin from numerous non-muscle cells in a variety of species can also self-assemble into short bipolar synthetic filaments (see 67, 68, 69).

Our current understanding of the self-assembly of myosin, which I have reviewed, is an elaboration of the earlier basic conclusions on the properties of "crystallized" myosin made in Szeged by Albert Szent-Gyorgyi the universally beloved Prof, in whose memory and honor this article is written.

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CLOTTING OF PROTEINS BY EXTRACELLULAR TRANSGLUTAMINASES *

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Transglutaminases are a family of transamidating enzymes present in both extracellular and intracellular environments in eukaryotes. Once they become activated from a zymogen or some other inactive configuration, these enzymes catalyze formation of γ-glutamylamine adducts of selected glutaminyl residues in proteins, but do not utilize free glutamine as substrates. The primary biological function of transglutaminases seems to be the cross-linking of proteins by N^{ε}-(γ-glutamyl)lysine bridges (1), although the incorporation of certain naturally occurring small amines into proteins has also been demonstrated (Fig. 1). These intriguing enzymes, first described by Heinrich Waelsch (2), are found throughout the animal kingdom and there is recent evidence for their occurrence in plants as well (3).

As noted for a variety of cell types [e.g., fertilized sea urchin eggs (4), murine erythroleukemic cells (5), macrophages (6), keratinocytes (7)] the intracellular enzymes - and often there are a variety of different forms of transglutaminases even within a single cell (1) - are developmentally regulated. Expression and distribution between soluble and membrane-bound activities may change during ontogeny or malignant transformation (8), and also in response to agents such as retinoids,

* *In memoriam Albert Szent-Györgyi*

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hormones and phorbol myristyl acetate by as yet unknown mechanisms (9). Conversion of the enzymes to active forms requires Ca^{2+} and might be promoted by Ca^{2+}-calmodulin (10); limited proteolytic cleavages can also facilitate the unmasking process (11). Cystamine (12), Zn^{2+} (13) and GTP (14) are some of the intracellular constituents that can inhibit transglutaminases; the first two of these block the cysteine thiol group at the active site.

Extracellular transglutaminases are involved in the clotting of blood, and also seminal fluid in certain species. For example, fibrin stabilizing factor or Factor XIII is the last zymogen to become activated in the human blood coagulation cascade (13,15). As depicted in Fig. 2, molecular details of its conversion are sufficiently understood to reveal some exquisite regulatory processes involved in the generation of transamidating activity. This cross-linking potency, though relatively specific for fibrin, must not arise in the circulation except as part of the clotting process; thus timing of the rate of production of Factor $XIII_a$ and sequestration of this enzyme to the clot lattice is a physiological necessity. Normally, there is negligible transglutaminase activity in blood plasma or serum.

A most rewarding approach for probing polymerization of protein substrates by transglutaminases is inhibition by small primary amines (16), with concomitant attachment of the pseudo-donor molecules to the same γ-glutaminyl residue of the acceptor protein which otherwise would participate in formation of N^{ε}-(γ-glutamyl)lysine bridges (Fig. 1). The enzyme-directed and site-specific labeling with amines, particularly with dansylcadaverine, proved to be of great advantage in the identification and analysis of protein substrates for transglutaminases in biological

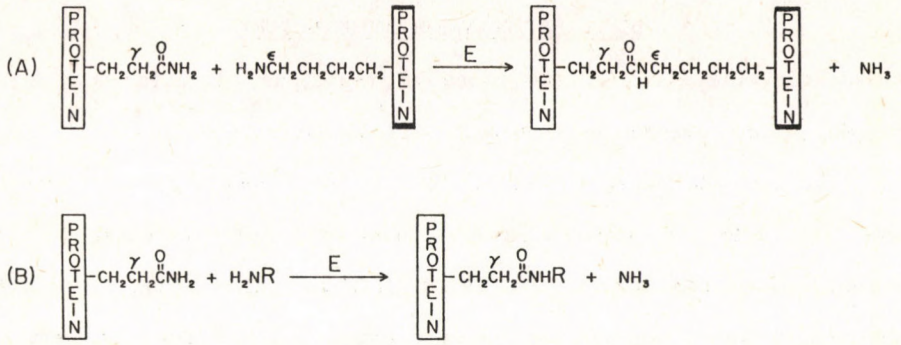


Fig. 1. Two biological reactions catalyzed by transglutaminases: (A) cross-linking of proteins, creating N^{ϵ}-(γ-glutamyl)lysine side chain peptides, and (B) incorporation of small molecular weight amines into γ-glutamine sites of proteins. Reactions (A) and (B) compete against each other.

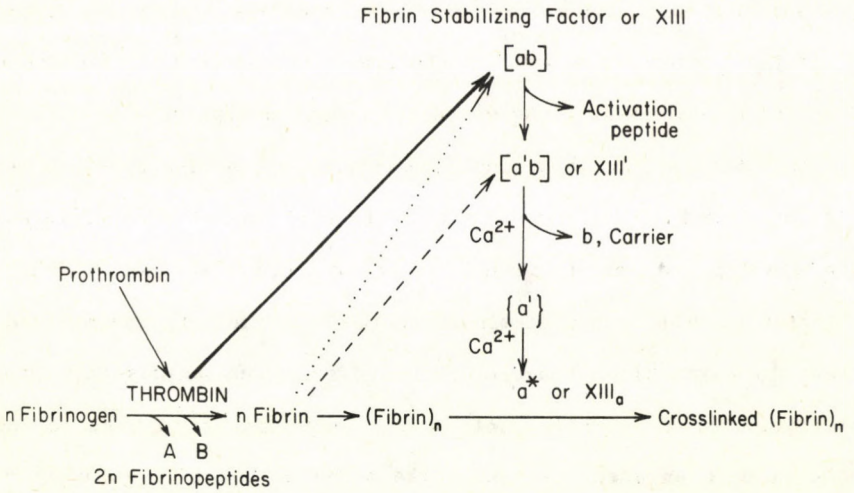


Fig. 2. Reconstruction of the physiological pathway of Factor XIII conversion and fibrin stabilization, emphasizing the "feed-forward" regulatory aspects by fibrin in promoting the reaction of Factor XIII with thrombin and in reducing the Ca^{2+} requirement for the dissociation of the hydrolytically modified [a'b] allozymogen to a* and b subunits (13).

systems.

DIFFERENT POLYMERIZATION MOTIFS

From clotting studies, two types of reactions, ligation and direct polymerization, emerged as seemingly general motifs.

(1) In human blood coagulation, Factor $XIII_a$ appears to roll over the network of fibrin aggregates, and, without ever being released into the medium, knits them together by N^{ε}-(γ-glutamyl)lysine bonds at specific end-to-end and side-by-side contact points (17). Even at very low frequencies (e.g., 1 mole per 100,000 to 400,000 g of protein), these covalent cross-links can exert a remarkable influence on the physical properties of the network (18), as illustrated by measurements of the elastic modulus (Fig. 3). Ligation, rather than cross-linking, would be a more apt term to describe the actions of Factor $XIII_a$ on fibrin. It may be surmised that the polymerization of membrane proteins mediated by the intrinsic transglutaminase of the human red blood cell represents a similar fusion of pre-existing protein assemblies (19,20).

(2) The other motif, which could be referred to as the direct polymerization of proteins, is exemplified by the clotting of lobster (Homarus) plasma (16,21) and, as discussed below, of rodent seminal fluids. In both instances, the transglutaminase is the sole clotting enzyme, and, in contrast to human blood coagulation where exogenous amines only prevent stabilization of the fibrin clot (i.e., conversion of a urea- or acid-soluble clot to an insoluble one), the added amines will actually delay the clotting time (Fig. 4). It is as if the transglutaminase, upon activation, would fish in a sea of soluble proteins, cross-linking only a few selected ones into polymeric structures. In lobster only a small

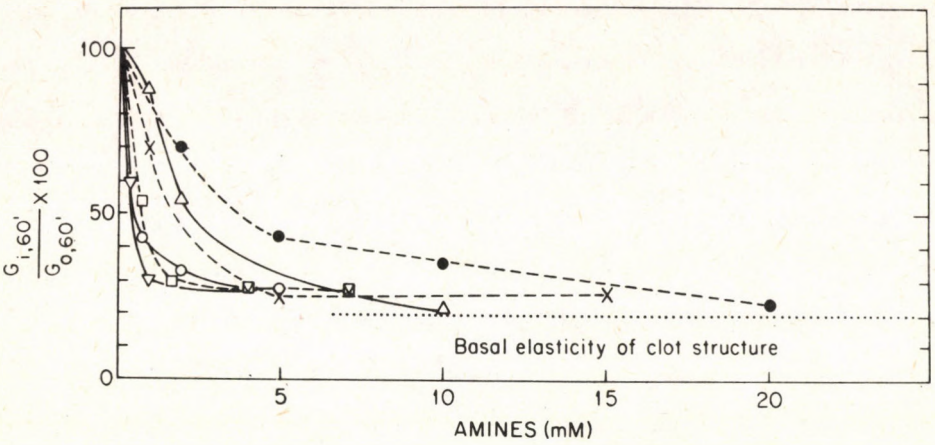


Fig. 3. Relative efficiency of inhibitors of fibrin stabilization in reducing the elasticity of normal plasma clots. The ordinate denotes the percentage of the elastic modulus at 60 min with the inhibitor ($G_{i,60'}$) in relation to no inhibitor added ($G_{o,60'}$). Dansylcadaverine (▽), mesitylenesulfonylcadaverine (○), dansylthiacadaverine (□), aminoacetonitrile (X), 2,4-dinitrophenylcadaverine (△), and hydroxylamine (●) concentrations are shown on the abscissa. The horizontal dotted line, referred to as the basal elasticity of clot structure, corresponds to an elastic modulus of 20% of normal (18).

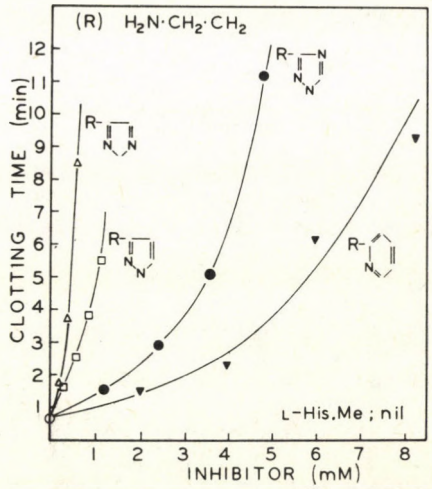


Fig. 4. Inhibition of clotting of Homarus plasma by β-substituted aminoethyl derivatives (16). Histidine methyl ester did not inhibit.

fraction of the total plasma proteins is precipitated in the form of a coagulum. Intracellular analogs for this motif may be the cross-linking of specific β-crystallin subunits in the lens, as may play a role in the generation of cataracts (22,23), and of involucrin in keratinocytes (24). Of course, in both situations the oligomeric products might eventually become fused to membrane constituents.

CROSS-LINKING OF PROTEINS IN RODENT COPULATORY PLUGS

When it is ejaculated into the upper vagina during coitus, the seminal fluid of rats, mice and guinea pigs coagulates almost instantaneously to form solid copulatory plugs, which evidently facilitate sperm transport to the sites of fertilization and perhaps play additional roles in reproduction (25-27). The proteins in rodent seminal plasma that act as the chief precursors of the seminal clot are derived from seminal vesicle secretion (SVS). These SVS proteins do not resemble fibrinogen, are not clotted by thrombin, and differ markedly between species. They are acted upon by Ca^{2+}-dependent enzymes secreted by the adjacent anterior lobe of the prostate or coagulating gland (CG) to produce coagula that are insoluble in 8 M urea plus 5 mM dithiothreitol. As first demonstrated in reconstituted guinea pig systems (28,29) the CG enzymes are apparently unique forms of transglutaminases that polymerize certain SVS proteins to highly insoluble precipitates that contain as many as 40 moles of N^{ε}-(γ-glutamyl)lysine bridges per 100,000 g of protein.

More extensive studies on the involvement of the secretions of various rat male accessory sex glands (Fig. 5) in the clotting of semen disclosed several novel features of the latter process. In the laboratory rat, the majority of the bulk proteins in SVS are coagulated by extremely active

transglutaminases in CG secretion that can be separated by agar gel electrophoresis into two forms that are distinct from Factor $XIII_a$ or liver transglutaminase. Three smaller (8-16 kD) monomeric SVS proteins, together with a series of larger SVS proteins (present in the secretion as mixtures of monomers and also homo- and hetero-polymers linked by disulfide bridges) are collectively polymerized into the seminal clot (25,26,30-33). The isodipeptide N^{ε}-(γ-glutamyl)lysine could not be detected, following exhaustive proteolytic digestion, in unclotted rat SVS proteins, whereas the coagula contained more than 9 moles of the isodipeptide per 100,000 g of protein, which almost certainly represents a far from maximal possible value for the extent of cross-linking (26).

In confirming the report of Beil and Hart (34) that sialoglycoproteins in rat bulbourethral gland (BUG, see Fig. 5) secretion accelerates the clotting of SVS proteins by CG enzymes in vitro, it was discovered (30,35) that low concentrations of certain macromolecular polyanions (poly-L-glutamate, poly-L-aspartate, poly[A]) exerted comparable stimulatory effects on the transglutaminase-mediated coagulation process. Since these macromolecular polyanions could not conceivably act as transglutaminase substrates, it was proposed that they enhanced coagulation by specifically interacting non-covalently with some of the SVS protein substrates so as to render certain reactive glutaminyl and/or lysyl residues more available to the active sites of the enzymes. The finding that poly-L-glutamate increased the covalent incorporation of putrescine into proteins of the clot and clot liquor, and also into SVS proteins whose lysyl residues had been blocked by chemical methylation, but not into N,N'-dimethylcasein, is in line with this hypothesis (30,35).

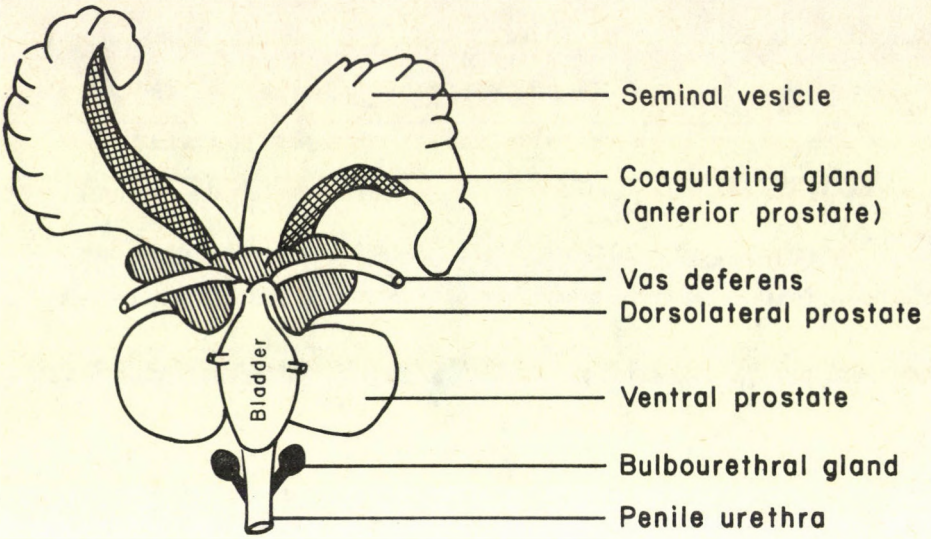
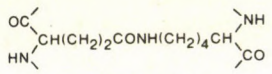
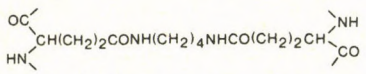


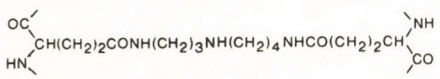
Fig. 5. The male accessory sex glands of the laboratory rat.



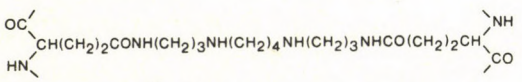
ε-(γ-glutamyl)lysine



N^1,N^4-bis(γ-glutamyl)putrescine



N^1,N^8-bis(γ-glutamyl)spermidine



N^1,N^{12}-bis(γ-glutamyl)spermine

Fig. 6. N,N-bis(γ-glutamyl)polyamine cross-links between proteins formed by transglutaminase action (40).

Rat seminal plasma is notoriously rich in the polyamines spermidine and spermine (36), which are present at roughly 5 mM in the secretions of the ventral and dorsolateral lobes of the prostate, but only to a negligible extent in SVS and the CG and BUG secretions (Fig. 5). (Polyamines are found in normal blood plasma and many other body fluids in lower than micromolar levels.) Tabor and Rosenthal (37) detected spermidine and spermine in hot 6 N HCl digests of rat copulatory plugs. Spermidine and spermine (and their biosynthetic precursor putrescine) are excellent substrates for all animal transglutaminases that have been examined. Studies on model systems (38) and on the coagulation of rat SVS proteins by CG secretion (39) revealed that polyamines can become covalently attached to proteins in the form of the corresponding N-mono-γ-glutamyl-polyamine derivatives, which then, as a result of the remaining primary amino group of incorporated polyamines also acting as an amine donor substrate, can react with a glutaminyl residue on another polypeptide chain with resultant formation of N,N-bis(γ-glutamyl)polyamine cross-bridges (40), as illustrated in Fig. 6. Rat SVS proteins that were coagulated by CG secretion in the presence of 0.13 mM spermidine and spermine were indeed shown to contain cross-links comprised of N,N-bis(γ-glutamyl) derivatives of these polyamines as well as of N^{ε}-(γ-glutamyl)lysine (39). Formation of such polyamine-containing bridges between proteins would be expected to occur during the production of rat copulatory plugs under natural coital circumstances because of the presence of spermidine and spermine in seminal plasma noted above. Whether this cross-linking of proteins by polyamines contributes significantly to the extreme insolubility of the rat seminal coagulum is not known. It is worth noting, however, that spermidine or spermine at 5

mM attenuate the clotting of rat SVS proteins by CG secretion in vitro, presumably because the polyamines compete with protein lysyl residues as amine donors in transglutaminase-catalyzed reactions. Perhaps the extensive secretion of spermidine and spermine by rat ventral and dorsolateral prostate is of adaptive biological value in preventing too explosive deposition during the ejaculatory process of clumps of coagulated protein in the male urethra which might compromise the delivery of urine (26,41).

Transglutaminase-mediated production of N,N-bis(γ-glutamyl)polyamine bridges between polypeptide chains was first demonstrated in the rat semen clotting system (39), which operates naturally in the extracellular milieu of the vaginal barrel. Does the formation of comparable polyamine cross-links between proteins occur intracellularly in any living animal cells? Beninati *et al.* (42) isolated tiny quantities of N^1,N^{12}-bis(γ-glutamyl)spermine from proteolytic digests of acid-precipitated fractions obtained from homogenates of rat livers that were previously freed from blood by perfusion in situ. There are reports of apparent covalent attachment of putrescine to nuclear proteins in regenerating rat liver (43), and of spermidine to a 30 kD intracellular protein in developing sea urchin eggs (44), although the precise nature of the linkages of the polyamines to proteins was not rigorously established.

Post-ejaculatory clotting of semen is evident in certain higher primate species as well as in rodents. In rhesus and African green monkeys, for example, the semen rapidly coagulates into hard lumps, and rhesus SVS is clotted by the secretion of the cranial (but not the caudal) region of the prostate gland (45). Immediately after it is expelled from the urethra, human semen appears as a whitish, viscous gel that then

liquifies within about 20 min at room or body temperature as a result of digestion of insoluble gel proteins by various proteinases and peptidases in seminal plasma that are derived from prostatic secretion (26). The main insoluble component of the human seminal gel is a high molecular weight protein originating from SVS (46,47). The epithelium of the human prostate secretes a transglutaminase with rather unusual properties (48), and also strikingly large quantities of spermine (41). Transglutaminase activity is demonstrable in dialyzed human seminal plasma (49,50). However, no evidence has been obtained as yet that the insolubility of the human seminal gel is related to the presence of either N^{ε}-(γ-glutamyl)lysine or N^{1},N^{12}-bis(γ-glutamyl)spermine cross-links between proteins.

We wrote this paper in loving memory of Albert-Szent Gyorgyi, a great friend to both of us and whom we revered as Prof.

ACKNOWLEDGEMENTS

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Abbreviations: SVS = seminal vesicle secretion; CG = coagulating gland; BUG = bulbourethral gland

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BOOK REVIEWS

BIOLOGICAL, BIOCHEMICAL AND BIOMEDICAL ASPECTS OF ACTINOMYCETES edited by G. Szabó, S. Bíró and M. Goodfellow. Symposia Biologica Hungarica. Vol. 32, Part A and B, Akadémiai Kiadó, Budapest, 1986, pp. 884

The Proceedings of the Sixth International Symposium held in Debrecen, Hungary, in August 1985, summarizes the biological, biochemical and biomedical aspects of Actinomycetes. The symposium was attended by 500 participants from 27 countries, 25 of them from Hungary. The camera-ready reproduction of the 121 complete papers read at lectures and mini-symposia and also all the abstracts are found in two volumes. It is published as the 34. volume of the FEMS Symposium series.

Meetings of this kind are held every third year and these conferences provide a great opportunity to make personal contacts with scientists who are engaged in studying the various aspects of Actinomycetes. Although most of the experimental works presented in this volume have been previously, or will be subsequently published, the volume serves a useful purpose by offering a general survey of the present state of Actinomycetes research. This topic is of considerable interest for biologists, physicians, pharmacologists, microbiologists, taxonomists, biochemists and biotechnologists both in industry and in academic research.

Papers are divided into nine areas: genetics, physiology, biosynthesis of secondary metabolites, biochemistry, morphology and ultrastructure, taxonomy, pathogenicity and immunology, ecology and epidemology, differentiation. Obviously such a collection of papers cannot provide a comprehensive review of each area, so selected examples are provided. Judging only by the number of papers presented at the symposium on various topics a shift of interest towards the genetics of Actinomycetes can be observed. At former Actinomycetes conferences

papers on the pathogenecity, immunology, epidemiology and ecology of Actinomycetes were represented in relatively higher number.

The invited speakers are the representatives of the most active laboratories of their own field of research.

Majority of the papers emphasize the results of their own laboratories. Only a few speakers, D.A. Hopwood, T. Beppu, M.J. Bibb, and S.T. Williams presented reviews in selected fields. The papers presented by invited speakers mostly seem to be a collection of interesting research reports because Actinomycetes consequently have very efficient biological activities and are used in numerous biotechnological processes as the producers of primary and secondary metabolites, enzymes, etc. This is why this book is recommended not only for those working with different strains of Actinomycetes, but for all biologists interested because "Actinomycetes are really beautiful" as Professor Szabó declared in his closing remarks of the Symposium.

A. Szentirmay

ELEMENTS OF BIOPHYSICS (sixth revised edition) edited by I. Tarján and Gy. Rontó, Medicina Könyvkiadó, Budapest, 1987, 362 pages (ISBN 963 241 421 1)

This book is an overview a pulling together of the subjects of biophysics into a handbook with primary aim to serve as the basis of a course for medical students. Although the text is intended introductory it is expected that readers will have a background knowledge in high school physics, chemistry, biology and mathematics. In addition to the descriptive review of fundamentals of biophysics special emphasis is laid on biological and medical applications. From this point of view the book outranges the average coursebooks and handbooks of biophysics.

The material has been organized in seven chapters.

1. Structure of matter, molecular basis of unity of structure and function,
2. Physical basis of medical applications of radiation,
3. Microscopic and submicroscopic methods for the investigation of biological structures,
4. Transport phenomena. Thermodynamics of vital processes,
5. Bioelectronics,
6. Biophysics of biological excitation and stimulus,
7. Information and control theory. Fundamentals of biokibernetics.

The revised edition includes additional material compared to the previous one in several topics. Paragraphs with more detailed information are those dealing with dosimetry, luminescence, radiation damage, biomedical applications of radioactive isotopes, analytical application of X-rays, physical methods for structural analysis, laws of diffusion, thermodynamics of biochemical processes, electrocardiography and computers.

Numerous photographs, drawings and diagrams are included to illustrate the text. The reader can find 15 tables about the SI system, physical constants. At the end of the volume a short subject index is also provided.

This book will be of interest not only to medical students but also to physicians, biochemists, transport physiologists, cell biologists and others involved in biological - biophysical research and physicists seeking an overall orientation in modern biophysical problems and medical applications.

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PLANT LECTINS - BIOLOGICAL FUNCTIONS (INVITED PAPER)

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The first plant lectin was discovered by Stillmark nearly a hundred years ago by finding a substance in the aqueous extracts of *Ricinus* seeds which agglutinated animal erythrocytes (1). Most seeds examined since Stillmark's pioneering studies were found to contain similar agglutinating proteins although their reaction with different erythrocytes showed a definite species-dependency (2). Some of these agglutinins were specific for human blood types within the ABO or MN blood groups (3,4), and to emphasize this specificity, Boyd and Shapleigh (5) called the haemagglutinins as lectins (from the latin word legere = to select).

The most general definition of lectins is based on the observation that agglutination can be inhibited by sugars in a very specific manner (6,7). Accordingly: lectins are proteins (or glycoproteins) of non-immuno-globulin nature capable of specific recognition of and reversible binding to carbohydrate moieties of complex glycoconjugates without altering the covalent structure of any of the recognized glycosyl ligands (8). This carbohydrate specificity can be most conveniently established by the Landsteiner hapten-inhibition technique (9,10) and accordingly the monosaccharides specifically reacting with lectins were divided into four classes based on their configuration at C-3 and C-4 of the pyranose

Abbreviations: CRM, cross-reacting material; IP_3, inositol-triphosphate; PHA, Phaseolus vulgaris; WGA, wheat germ agglutinin

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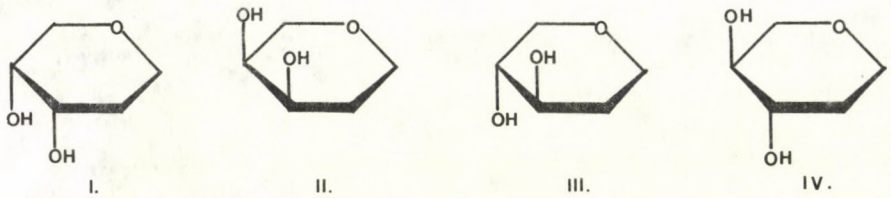
ring (11):



I. II. III. IV.

Thus concanavalin A (con A) which reacts with mannose and glucose belongs to group III, soyabean lectin reacting with N-acetyl-galactosamine and galactose to group II and the L-fucose binding lectins are classified as group I. In oligo- or polysaccharides lectins usually react with the non-reducing terminal sugar. In this terminal sugar residue the specificity of the lectin tolerates very little variation at C-3 or C-4, but C-2 appears to be less critical (10).

The great upsurge of interest in lectins in the last thirty years was the result of two fundamental observations on their biological function. Thus Nowell discovered in 1960 that the lectin from the seeds of *Phaseolus vulgaris* (PHA) had a strong mitogenic effect on peripheral lymphocytes (12). The other observation which had a stimulating effect on lectin studies was the recognition by Aub and co-workers that transformed cells had a greater affinity for lectins than the cell lines these originated from (13). Both of these functions are due to the specific reactions of sugar residues on the cell surface with the appropriate lectins. As the composition of the cell surface changes throughout the life cycle of the cell and during transformation it is not surprising to find that these changes are also reflected in the cells' reactivity with lectins.

As lectins are nowadays easily obtained in great purity by affinity chromatographic techniques based on their specific binding to sugar ligands covalently linked to appropriate supports, their use in chemical, biochemical, cell biological, clinical, etc. applications in widespread. For example, bound to chromatographic supports lectins are very convenient tools for purifying glycoproteins and glycosylated nucleic acids.

Lectins are also widely used for structural studies on soluble and membrane-bound glycoconjugates. Furthermore by the application of suitable lectins single cell lines can be separated from complex mixtures and transformation or mutation reactions of cells can also be followed easily.

Although originally lectins were discovered in plants they are now known to be one of the fundamental building stones of living matter (14,15) and their role in cell to cell communication, signalling and cellular adhesion is recognized as uniquely important in all forms of life. However due to their complexity and widespread occurrence in Nature the present review will be restricted to lectins only from higher plants and their biological functions.

Functions in plants

Lectins are ubiquitous in the Plant Kingdom. However usually the seeds are the richest source of lectins and particularly those from the families of *Leguminosae* and *Graminaceae*. Indeed, in the seeds of some plants such as the tepary bean (*Phaseolus acutifolius*), the lectin is the most abundant protein (16). Lectins of lectin-like and immunochemically cross-reacting materials (CRM) are also found in other tissues of plants. However the concentration of tissue lectins of CRM-s is usually much less than that of the corresponding seed lectins (17-19).

In *Leguminosea* the seed lectins are located mainly in the cotyledons, while in the *Graminaceae* in the embryo. The biosynthesis of the cotyledonary lectins runs parallel with that of the seed storage proteins (17). Moreover most of the lectin; just as the storage proteins, are located in the protein bodies of the cotyledons (20).

In *Dolichos biflorus* at least three different lectins are found (21-24). There is a lectin in the seed which is specific for N-acetyl-D-galactosamine and a CRM glycoprotein in the stems and leaves of the same plant whose sugar specificity and immunochemical reactivity is similar but not identical

with that of the seed lectin. In addition a third lectin is to be found in the roots whose sugar specificity is identical with that of the seed lectin although the two lectins have different amino acid composition and immune-reactivity. The CRM lectins biological function(s) in unknown while the root lectins most likely have a role in the various interactions between plants and bacteria. Of these the most important is the symbiotic reaction between the roots of plants of *Leguminosae* and the nitrogen-fixing *rhizobial* bacteria.

It has been suspected for some time that as because of their varied sugar specificity lectins can distinguish between various cell types and react with them selectively, they are probably one of the main agents in the defence of the plant against attacks by pathogenic microorganisms. Thus for example fluoresceine-labelled wheat germ agglutinin (WGA) was shown to bind to the hyphal tips and septa of *Trichoderma viride*. As in the mature hyphae the chitin is no longer accessible the WGA does not bind. Moreover WGA inhibits spore germination and the growth of the fungus by interfering with chitin biosynthesis. Finally all these effects of WGA on the fungus could be effectively inhibited by chitotriose (25). The binding of WGA to the hyphal tips and septa and young spores is known to occur generally with a number of other chitin-containing fungi (26). Other lectins including those from soyabeans and peanut also display similar reactivity (25,26). Potato lectin will similarly inhibit hyphal extension and spore germination of *Botrytis cinerea* (27) and WGA binds to mycobionts from lichen fungi (28), while soyabean lectin inhibits mycelial growth of *Phytophthora megasperma* (29). Thus these results appear to support the suggestion that lectins protect plants against pathogenic fungi at stages of vulnerability during imbibition, germination and early seedling growth (25,26).

Plant resistance to pathogens may also be due to lectins recognizing and reacting with potentially harmful bacteria (30). For example, the potato lectin reacts with the saprophytic but not with the pathogenic species of *Pseudomonas solanacearum* (31). Similar observations were made with PHA

(32) although there is some doubt now about the general applicability of these findings (19). In fact in some instances the results appear to suggest that lectins do actually promote pathogenesis (30). One particularly interesting example is found with a lectin-like protein from the sugar cane which binds the toxin from the fungus, *Helminthosporium sacchari* (33).

Lectins occasionally may defend plants against beetle predators. Thus resistance to *Callosobruchus maculatus* was conferred onto artificial pellets when PHA was incorporated in them (34). By immunofluorescence studies it was shown that the lectin bound to the gut of the larvae and this probably interferred with food absorption.

Arguably the most important interaction of plant lectins with exogenous cellular receptors is the legume root-*Rhizobium* host-symbiont relationship (35). The resulting N-enrichment of soils has long been exploited in agricultural practice though our understanding of the underlying biochemical and genetic mechanisms is still far from complete (17,19,36,37). One of the best studied examples of the symbiotic reaction is that between *Rhizobium trifolii* and clover roots (36). Although the root hairs of the clover plant bind many bacteria in a weak and non-specific way, very large numbers of the right *Rhizobium* attach themselves to the roots in a firm and host-specific manner. In the first, docking stage the fibrillar capsule of *R. trifolii* makes a physical contact with globular aggregates on the cell wall of the root hairs. The attachment is through the multivalent clover lectin, trifoliin A, which forms a bridge between two immunochemically cross-reacting polysaccharide antigens, one on the surface of the bacteria and the other on the cells of the root hairs (38,39). This step is specifically inhibited by the haptenic sugar, 2-deoxy-D-glucose. Although this sugar prevents the binding of *R. trifolii* to clover roots, it has no effect at all in other plant-*Rhizobia* systems. The number of receptor sites on root hairs is the highest at the tips where the bacteria are actually bound and decreases towards the base. In phase II of the symbiosis the bacteria secrete a fibrillar material

which anchors the bacteria to the root hairs and this probably is also responsible for inducing root hair curling and the penetration of the bacteria through the cell wall. Although bacterial attachment is a prerequisite step of symbiosis it is by no means the only requirement for the success of the infection. It has become clear that root hairs are only transiently susceptible to infection and the receptor polysaccharide on the bacteria is also transient and its presence is controlled by the root hair attachment (roa) genes which occur on large transmissible plasmids. These genes, when transferred to non-infective mutants make these bacteria bind to root hairs but still non-infective. Thus in addition to cellular recognition mediated by lectins for a successful symbiotic reaction other steps are needed, such as a number of attachment-induced *de novo* synthetic reactions, de-repression of specific genes both in the bacteria and the host cells and a general turn-on of the production of cellular materials necessary for the formation of the infection thread, bacterial penetration and finally the nodulation. Thus the lectin-mediated recognition is but a first step in a multistep reaction and followed on by a continuous exchange of signal molecules between the host and the bacteria covering the whole period of the infection (37).

Despite the great advances in our understanding of the clover - *R. trifolii* symbiotic reaction there are doubts about the general applicability of the model suggested by Dazzo and co-workers (38,39). Thus it will still have to be established if the receptors on both the host and the bacteria are present in all symbiotic reactions at exactly the right time. Moreover it has not been shown generally that the specificity of the host lectin is always proper for the recognition of the appropriate *Rhizobium*.

Lectins probably take part in recognition reactions between different cells of the plants and particularly in pollen-pistil interactions where recognition by lectins may be the chief pre-requisite step determining the success of pollination (40).

Lectins may form cross-links between components of the plant cell wall and thus take part in auxin-mediated cell wall elongation (41). However these claims have not been substantiated by later work (42).

Finally there is a great deal of indirect evidence to support the suggestion repeatedly put forward by great many workers that the seed, tuber, bark etc. lectins may function simply as storage proteins (17,19). Indeed as there are usually large amounts of lectins found in these tissues and their synthesis, location and developmental control of their appearance and disappearance during germination in the seed show great similarities to those of other well-recognized storage proteins the balance of evidence tends to support such a role.

Effects of plant lectins on animal cells

Agglutination. This is one of the most characteristic and important reactions of those plant lectins which have at least two carbohydrate-binding sites. However the relationship between the extent of agglutination and the amounts of lectin bound by the cells is not always straightforward. In most instances agglutination and its extent is dependent on the properties of the cell surface, the metabolism of the cells, the characteristics of the lectin and various physical factors. By manipulating some of these determinants individual cell lines can be separated from complex cell mixtures with the help and the exploitation of agglutination reactions.

In most instances the extent of agglutination can be increased by an appropriate pre-treatment of the cells with various enzymes. Thus as agglutination is usually the result of a reaction between the lectin and cell surface glycolipids, the removal by proteolysis (e.g. trypsin) of peptide chains protruding from the membrane lipid layer will facilitate a closer contact (and increased agglutination) between the lectin and the cells. The removal of surface sialic acid residues by neuraminidase may uncover new receptors in the cell membrane and thus increase agglutination. Aggregation of the

lectin may also increase cell agglutinability by increasing the extent of the surface which can be covered by the larger size aggregated lectin. Thus the soyabean lectin after polymerization with glutaraldehyde is a more potent agglutinin than it is in its protomeric form (43). The opposite happens when the originally tetravalent con A is made divalent by converting it to the 500 times less active dimeric succinyl-con A derivative(44). On the other hand the modification of carbohydrate side-chains usually has no effect on the agglutinating properties of lectins (45).

There may also be differences in the extent of agglutination of normal and transformed and embryonic or mature cells. However this is not always true and even when it occurs its biochemical mechanism is not clear.

Finally in addition to animal cells, cells from micro-organisms, fungi, algae, higher plants and others, can be similarly agglutinated. In some instances even subcellular particles can be agglutinated by lectins.

Mitogenic stimulation of lymphocytes

As mentioned previously, Nowell's discovery that PHA stimulated the quiescent and non-dividing lymphocytes to blast formation and mitosis had a great effect on lectin research generally (12). It is recognized nowadays that under favourable conditions most lectins (if not all) are mitogenic (46).

Although for a while it was believed that lectins react only with T-lymphocytes, it was soon shown that for example pokeweed mitogen stimulated both T and B lymphocytes (47). More recently it was also recognized that both WGA and the lentil lectin may stimulate both types of lymphocytes under favourable conditions (46). Moreover WGA may inhibit the lymphocyte transformation reaction stimulated by other lectins (48). Similar inhibition could be achieved with the tomato lectin which in itself is not mitogenic (49).

The great practical advantage in the use of lectins or mitogens for lymphocytes, in contrast to the similar transformation caused by antigenic stimulation, is that lectins are polyclonal reagents and as they react with almost all lymphocyte clones the extent of the mitogenic transformation may reach as much as 80 per cent of the whole lymphocyte population (50). As in this reaction both the lectins and antigens bind to the same receptors the more extensive transformation (51) of the lymphocyte preparations by lectins facilitates the study of the mitotic reaction generally.

In the mitogen-lymphocyte reaction the lectin will first bind to cell surface glycoconjugates. However through this binding a number of intracellular metabolic reactions will also be affected. One of the first measurable effects of the lectin binding to the lymphocyte is an increased membrane permeability and phospholipid metabolism. Both the methylation of phospholipids and the degradation of the methylated phospholipids are accelerated (52). Signal molecules, such as arachidonic acid and its derivatives (prostaglandins, thromboxanes, etc.) are then produced by the action of phospholipase A_2 and the importance of these in the mitogenic transformation is now generally accepted. From the hydrolysis of the membrane phosphatidyl-inositol 4,5-biphosphate (PIP_2) potent secondary messengers are also being produced. Thus one of the hydrolysis products, inositol-triphosphate (IP_3), facilitates the mobilization of intracellular, bound Ca^{2+} and this increases the free cytosolic Ca^{2+} concentration (53), while the other product, diacylglycerol activates the Ca^{2+}- and phospholipid-dependent protein kinase C enzyme (54). Thus in T lymphocytes after treatment with PHA the activity of protein kinase C increases in both the cytosol and the membranous fraction (55). Similar observations were made with WGA. However with WGA there was also a need to use sub-mitogenic amounts of phorbol-12-myristate-13 acetate for effecting lymphocyte transformation (56). The lectin-induced membrane reactions also lead to other intracellular reactions including the acetylation of histones and the phosphorylation of nuclear proteins. The consequent de-repression of DNA leads to

increased RNA and protein synthesis. As a result the lymphocyte size increases and other morphological changes ensue while the production of interleukin-2 (57), γ-interferon (58), B-lymphocyte factors (59), granulocyte-macrophage factors (60) and other growth-stimulating or inhibitory factors is stepped up. About 48 h after the original lectin-stimulus DNA-synthesis and consequently mitosis starts up. About 24 h later the fully differentiated B-lymphocytes begin to produce immunoglobulins while the T cells become cytotoxic (46).

The extent of mitosis may occasionally be influenced by treatment of the cells with various enzymes. Thus for example murine lymphocytes need a pre-treatment with neuraminidase (61) when soyabean lectin is used as a mitogen. However as even non-mitogenic lectins are bound to the lymphocytes, it is not clear that the reaction of which component(s) of the membrane with the lectin is the necessary pre-requisite step for the mitotic transformation. All the same more recent evidence shows that for mitogenicity lectins have to be able to produce a set of lymphocytes with functional receptors for interleukin-2 and also other sets of lymphocytes (usually at higher lectin concentrations) which can synthesize and secrete interleukin-2. In the second proliferation phase of the mitogenic stimulation the interleukin-2 produced by lymphocytes reacts with the lymphocyte set containing the interleukin-2 receptors and in these cells DNA-synthesis and mitotic division begins. Thus the differentiation and the proliferation stages of the mitosis of the cells are clearly separable. Other factors may also promote the mitotic reactions. Thus it is possible that interleukin-1 produced by lectins from macrophages helps the synthesis and secretion of interleukin-2 and consequently indirectly interleukin-1 promotes lymphocytes mitosis.

Lymphocytes, after treatment with lectins, may also produce suppressor cells capable of inhibiting all the biological activities of both B and T lymphocytes. These cells probably control the proper *in vivo* functioning of the immune system (46). The suppressive function is probably mediated by soluble factors released from these suppressor cells (62).

One of these factors inhibits the proliferation of T lymphocytes (63) while the other reduces the immunoglobulin production by B cells (64). Thus it is possible that lectins influence lymphocyte function both in a positive and a negative direction and this may be the result of stimulating different regions of the lymphocyte surface (65).

The lectin-induced suppressor cells have important applications in medical-clinical practice. The measurement of suppressor T-cell function after lectin stimulation in diseases which are caused by deficiencies in the functioning of the immune system can give indications about the immune competence of the patients (66). Because of the immune suppressive effects of PHA the survival of skin allografts was significantly extended over that of PHA-free controls (67). The survival time was even more increased if both the donor and the acceptor rats were pre-treated with PHA (68). It was also shown recently that the suppressor B-cells formed from human peripheral B-lymphocytes by PHA-treatment inhibit both the allogeneic mixed lymphocyte reactions (MLR) and DNA-synthesis (69). This reaction is not mediated by T lymphocytes and is a direct effect on B-cells. PHA also inhibits thyroglobulin-induced autoimmune thyroiditis if the lectin is applied intravenously before the thyroglobulin (70). Similarly the presence of PHA in the diet is immune suppressive and inhibits the production of antibodies to other immunogenic food proteins (71,72).

The cytotoxic T lymphocytes which normally kill target cells based on a specific recognition by the effector cells of major histocompatibility complex antigens lose their antigen specificity in the presence of con A or other mitogenic lectins. The lectin cross-links the effector and the target cells and because of the proximity the cytolytic activity of the effector cells lyses the target cells (73). Under similar conditions some lectins like WGA may induce the killing of tumour cells by macrophages (74) by cross-linking carbohydrate receptors on the two types of cells. This reaction is very similar to the lectin-dependent lymphocyte cytotoxicity and both of these cytotoxic reactions may also occur *in vivo*. Thus for example previously applied *Griffonia*

simplicifolia I lectin protects mice innoculated with Erlich ascites cells from developing tumors (75). Lectins can also facilitate the phagocytosis of target cells. Thus in the rosettes formed between con-A-coated murine macrophages and erythrocytes the erythrocytes are phagocytosed by the macrophages after treatment with more lectin (76).

In allergic reactions histamine or other pharmacologically active amines are liberated from basophil or mast cells when these cells are exposed to a number of secretagogues. The liberated amines have strong and characteristic allergic effects on the surrounding tissues. In the classical allergic reaction the allergenic antigen binds to the specific IgE on the cell surface and forms bridges between the mast cells. Subsequently, in a way that is not well-understood, free Ca^{2+} -ion concentration increases in the cell cytosol and by an energy-dependent reaction histamine is secreted without any damage to the mast cells. This exocytotic reaction, the main biological function of mast cells, can also be induced by various lectins (71-79). It is however not clear yet if in addition to lectins the presence of other reagents, such as Ca^{2+}-ions, phosphatydil-serine, etc, are also needed (80) for the exocytosis, although it is known that there is no need to sensitize the animals with allergenic antigens previous to the preparation of the mast cells. Lectins can also inhibit the exocytosis induced by other secretagouges (79) and bind to both the carbohydrate receptors on the cells or the IgE bound to mast cells (81). The dietary PHA has an immediate and direct effect on gut mast cells. Moreover, on longer feeding with this lectin there is a significant increase in the gut anaphylactic reaction suggesting the formation and the involvement of lectin-specific IgE (82). There are indications that under similar conditions mice show type-I hypersensitivity reactions due to the synthesis of systemic IgE antibodies specific for the dietary PHA (83). Lectins such as PHA or jacalin (from *Artocarpus integrifolia*) dependent on their concentration may have different effects on IgE-production to simultaneously injected unrelated antigens. Thus at low lectin doses jacalin stimulates helper lymphocyte

function while at higher doses the lectin may be strongly immune-suppressive, reducing the production of the unrelated antigen specific IgE (84). This type of lectin-induced IgE suppression may have important applications in medical practice.

Lectins are known to mimic the effects of insulin on adipocytes and accordingly lectins like Con A, WGA, PHA and others stimulate the synthesis of triglycerides and the transport and oxidation of glucose in these cells (85-87). Similar effects were also observed *in vivo* (88). In contrast dietary PHA (and possibly soyabean lectin) reduces the blood insulin concentration within a very short time after reaching the small intestine (89,90) with the consequent increase in fat, carbohydrate and protein breakdown in the body.

It appears that both insulin and lectins (con A, WGA) bind to the same receptor(s) on the adipocyte (85). However the insulin-receptor complex after endocytosis ends up in the lysosomes where the insulin is degraded while the receptor is re-cycled to the membrane and then can bind another insulin molecule. Thus insulin affects the cells for a short time only and for a new stimulus a new insulin molecule's binding is required. In contrast the lectin-receptor binding is stable and to stop its effect the lectin has to be washed off with the appropriate specific sugar from the surface membrane. Thus the insulin receptor cannot be used to internalize the lectin by endocytosis (91).

Biological functions of dietary lectins

Most foods, and particularly those of plant origin contain lectins in different amounts. If these lectins survive the passage in the gut in significant amounts, by virtue of their reactivity with cells lining the gut, they may interfere with the normal digestive, absorptive and protective functioning of the digestive system. Although some lectins are inactivated by proper heat treatments a great number of vegetables, fruits, nuts and cereal germs all containing lectins are consumed uncooked (92,93). Moreover some dietary lectins

cannot be inactivated by heat treatments and thus even humans consume substantial amounts of lectins as part of their normal daily diet (92,93).

One of the most important and extensively studied dietary lectins is PHA. Bean seeds contain large amounts of PHA (about 15 per cent of the total protein) and rats fed on a diet containing 10 per cent bean proteins lose weight and die rapidly, within 2-4 days. It has been unequivocally established that the nutritional toxicity of kidney bean is caused by PHA and that the extent of the toxicity is dependent on the amount of PHA in the diet (89,90). Up to 90 per cent of the dietary PHA survives passage through the digestive tract and binds to the surface cells of the small intestine, such as the brush border enterocytes, goblet and other cells and damage their luminal membranes. Moreover PHA reduces the extent of N absorption from the small intestine and above a certain dietary PHA concentration the overall loss of N exceeds that eaten: the animal is then in a negative N-balance (94). This indicates that PHA does not only react with the gut (local effects) but that it also influences the body's systemic general metabolism with the catabolic effects dominating. PHA, after binding to the luminal surface, is endocytosed by both the enterocytes and goblet cells and modifies their basic metabolism (95). One of its most striking effects in the gut cells is the immediate near doubling of the mucosal *in vivo* protein synthesis rate (96) which uses up a large part of the dietary N and starves the body of its amino acid supply (96). The immediate turn-on of the protein synthetic apparatus of these cells is probably connected with the increase in mucosal polyamine content (Wallace, Grant, Bardócz & Pusztai, unpublished) which in turm precedes the observed lectin-induced cell proliferation and increased cellular differentiation (97).

A substantial part of the endocytosed PHA gains access to body tissues and fluids and about 5-10% of the dietary lectin (labelled with ^{125}I) is found in the circulating blood within 3 h of feeding while the absorption of the relatively non-toxic tomato lectin (98) is minimal (about 0.1%).

Similarly, PHA is much less toxic in gnotobiotic animals where the systemic absorption of PHA is greatly reduced (99). Thus in some way, not clearly understood at present, the extent of the nutritional toxicity of lectins is dependent on whether significant amounts of the lectin are systemically absorbed or not (99).

There are indirect indications that significant amounts of PHA are taken up by the body. In all animals, even in some ruminants, the systemic absorption of PHA produces a monospecific and exclusive high titre antilectin IgG response (71,72,89,90). This indicates that the gut s-IgA production is not sufficient to prevent the systemic absorption of the lectin. The dramatic *E. coli* proliferation in the small intestine may also be the result of a general lectin-induced interference with the gut immune system (100,101). The blood protein loss into the gut lumen caused by the anaphylactic reaction of the lectin with small intestinal mast cells is another manifestation of the widespread interference of the dietary PHA with the whole immune system (82,102).

PHA reacting with the gut hormone-producing cells or the systemically absorbed lectin interacting with endocrine tissues changes the body's hormonal balance (89,90,99). The immediate drop in blood insulin and increase in glucagon concentration have the net effect of accelerating the catabolic breakdown of fats, carbohydrates and proteins in the body. The breakdown of body lipid is particularly fast and although there is also muscle protein loss (96) the body's relative protein concentration is increased (99). Although the insulin concentration is low the rats are not diabetic, in fact quite the opposite, for after an overnight fast the blood sugar concentration in these rats becomes dangerously low (6-7 mg per 100 ml blood) indicating that the energy stores in the PHA-fed rats become almost exhausted. The results also show that the lectin probably influences the production of hormones by all endocrine cells and the observed changes in metabolism are the result of a new hormone balance induced by the dietary lectin. Changes in the weight, composition and structure of internal organs such as the

thymus, liver and especially the hypertrophy of the pancreas, all appear to give independent, though indirect support for the general systemic effects of lectins on the immune and the hormone-producing systems of the body.

It is known that some of the effects of dietary PHA are also observed with lectins from other plant foods (89,90,99). Although our knowledge of the properties of other dietary lectins is much less complete the main characteristics of the antinutritive effects of lectins generally on monogastric animals are becoming clearer.

Thus: 1. Significant amounts of dietary lectins are not broken down in the digestive tract.

2. Lectins bind to and disorganize the membranes of the brush border cells of the proximal small intestine, and as a result reduce the extent of food digestion and absorption, increase cellular protein and mucin synthesis and secretion, accelerate cell turnover induce hyperplasia.

3. Lectins increase the permeability of the small intestine for macromolecules and especially for the more toxic lectins with consequent effects on systemic metabolism and on the immune system.

4. Lectins shift the systemic hormone balance towards increased catabolic breakdown of body lipid, carbohydrate and protein.

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REGULATION OF MOLLUSCAN MYOSIN BY LIGHT CHAINS*

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In this paper I will discuss myosin based regulation of contraction that is exemplified by molluscan muscles and point out some of the features that make the study of these muscles particularly favorable to clarify the molecular mechanism of calcium control. Similarly to other muscles, contraction of molluscan muscles is triggered by calcium and the contractile system consists of actin, myosin and Mg-ATP. The unusual state is the resting state, to maintain it regulatory componets are required. In vertebrate skeletal muscles troponin-tropomyosin acting on thin filaments account for regulation (1). In a number of invertebrate muscles, vertebrate smooth muscles and non-muscle cells myosin itself is a regulated molecule, con - trolled by its subunits, the light chains. Calcium interacts directly with troponin C or with molluscan myosin so that the control system is no longer inhibitory. In smooth muscles and non-muscle cells, however, the calcium effect is an indirect one, phosphorylation of the light chain by the calcium activated myosin light chain kinase triggers contraction (cf. 2-6).

The unique feature of molluscan myosin is that it requires calcium for activity even in the absence of troponin-tropomyosin and that it has a high affinity specific calcium binding site that is saturated at 10^{-6}M free calcium concentration in the presence of milimolar amounts of magnesium (7). Molluscan myosin therefore is a regulated molecule that is

Abbreviations: S1, subfragment-1; RLC, regulatory light chain ; ELC, essential light chain; EDTA, ethylene diamine tetra-acetic acid

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able to sense the calcium released when the muscle is stimulated. The subunit structure of regulatory and non-regulatory myosins is very similar. Both types contain two heavy chains of about 200.000 daltons and four light chains of two kinds of weights somewhat below 20,000 daltons, the regulatory and essential light chains (RLC and ELC) (8). Like in vertebrate myosin the light chains of molluscan myosins are accosiated with subfragment-1 (S1) representing the globular myosin heads. The controlling role of these light chains have been established by following the calcium dependent functions such as calcium binding, actin activated Mg-ATPase or tension development after their removal (in case of the RLCs)(9-11) or after treatment with specific antibodies (in case of the ELCs) (12).

RLCs detach from the heavy chains of scallop myosin in the absence of divalent cations. One RLC is removed from scallop myosin by EDTA in the cold (9), both regulatory light chains dissociate at elevated temperatures (10). Regulation is lost upon removal of the RLCs. Such preparations consisting of the myosin heavy chains and ELCs no longer reguire calcium for activity, and the specific calcium binding sites are replaced by sites of lower affinity and specificity (10). The preparations without the RLCs lose their calcium dependent functions, they are desensitized. Without the RLCs the muscles can contract,but are unable to relax; ATPase activity is no longer inhibited by the removal of calcium. RLCs can be removed also from myofibrils (9) or skinned fibre bundles (11) and their contribution to structure and function can be studied at various levels of organization. Light chain dissociation is reversible. The original RLC content of myosin is readily restored in the presence of magnesium ions by incubation with a slight excess of the light chain. The reconstituted preparations fully regain calcium dependent regulatory functions; are resensitized.

Scallop myosin is a particulalry suitable material for the study of myosin-based regulation since its regulatory light chains can be made to release without denaturing the heavy chains. EDTA treatment works only partially with other

myosins, and the methods required for RLC removal denature the heavy chains. The RLCs of chicken and rabbit myosin have been removed with the aid of specific antibodies (13) or by limited chymotryptic digestion of the actomyosin complex (14); however, vertebrate skeletal myosin is unregulated. The light chains resist denaturation, remain functional after treatment with urea, guanidine-HCL, detergents or heat and may be obtained from different myosins. These foreign light chains readily hybridize with desensitized scallop preparations (15-18). The hybrids regain regulation if the source of the light chains was regulatory myosin. Light chains from vertebrate skeletal and lobster tail myosins do not restore regulation and calcium has no effect on the activity of the hybrids. Hybrids formed with gizzard light chain can be activated both by 0.1mM calcium or by phosphorylation (17). It is clear therefore that various RLCs have common attachment site(s) and contribute to regulation in a specific manner. The amino acid residues at sequences responsible for the differences between functional and non-functional RLCs, have not yet been identified.

RLCs of different species vary considerably. The sequence homology between scallop myosin RLCs (19, 20) and vertebrate skeletal RLC (21) is only about 30%. Scallop RLCs lack cysteine, an amino acid obviously not required for function. Several foreign light chains contain one or two cysteine residues that can be substituted without interfering with their regulatory function (8,15). These thiols therefore, may be utilized to introduce probes to myosin. Since cysteine has been found at different positions of the RLCs, the foreign hybrids are convenient tools for the study of light chain function. They have been utilized to identify domain-1 near the N-terminus as the divalent cation binding site (22,23) and helped to localize the regions of the light chains that are rearranged during rest, rigor and activity (24-25).

The function of the R-LCs as a regulatory subunit has been established directly since these light chains can be easily removed from and readded to scallop myosin. There is only indirect evidence for a regulatory role of ELCs. Removal

of ELC so far requires procedures that denature the heavy chains. Although ELC can be exchanged with ELC of the medium (26), ELC free preparations of regulatory myosins in native conditions have not yet been obtained. A regulatory role for ELC is suggested by the findings that specific antibodies directed against the ELC (both IgG and Fab fragments) desensitize myosin (12). In addition, several evidence indicates that ELCs and RLCs interact and this interaction is different at rest than in rigor or activity.

The light chains occupy the neck region of scallop myosin (27,28). Removal of light chains is accompanied by losses of material from the proximal narrow part of the myosin heads as seen by electron microscopy of rotary shadowed preparations, and this is the region where specific polyclonal antibodies against ELC and RLCs are bound (26). The importance of the head-rod junction in regulation is indicated by the behaviour of the proteolytic fragments of myosin. Myosin, single headed myosin and heavy meromyosin (the soluble double headed fragment of myosin) are regulated molecules. In contrast, S1 prepared in the presence of divalent cations, is unregulated and is fully active in the absence of calcium although it retains the light chains and binds calcium (29). Light chain binding is retained by a small 12,000-14,000 dalton weight tryptic fragment of S1 (30). This "regulatory peptide", like intact myosin, binds RLC only in the presence of divalent cations. The RLCs and the peptide mutually protect each other from digestion by proteolytic enzymes; free RLC is rapidly degraded by trypsin (30). S1 obtained from myosin in the absence of divalent cations contains no RLCs and only a fragment of the ELC (29). The S1 preparation is unable to bind light chains, is somewhat smaller (28,31) lacking a small stretch of the heavy chain (ca.3000 daltons) from its C-terminus that participates in the headrod junctions. The importance of this region is further indicated that in its absence, the intrinsic fluorescence of S1 no longer responds to calcium (32). The "regulatory" peptide is part of the 24,000 dalton tryptic fragment of myosin and constitutes the C-terminal end of S1 (31). Digestion of

myosin with the proteolytic enzyme from Pseudomonas aeruginosa yields a fragment where the 24,000 dalton piece of S1 remains covalently linked to the rod and retains the light chains to form the "regulatory domain" (33).

Interaction between light chains is further indicated by findings that RLCs suppress the reactivity of ELC thiols (34), and inhibit the exchange of ELCs (26). In addition, ELCs and RLCs can be extensively cross-linked in myosin in rigor solutions (no ATP) by short (<10A) cross-linkers that are attached to foreign RLCs thiols at positions ca. 50 /Mercenaria RLC (23)/, 108 /gizzard (35)/, 129 and 156 /rabbit (21)/. These results suggest that ELC and RLC are closely spaced to each other on the myosin head along half or more of their length (24).

Photo-cross-linking studies have shown that the light chains move when the state of myosin in myofibrils is changed depending on the presence of ATP and calcium (25). The N-terminal third of the RLC cross-links with ELC only in the absence of ATP ("rigor") or in the presence of both ATP and calcium ("activating solution"). No cross-linking takes place in "relaxing" solutions (ATP but no calcium). When ELC is cross-linked with the N-terminal portion of the RLC, regulation is lost, and the system is locked in the "on" position. Rearrangement of the light chains is restricted to the N-terminal region of the RLC. The C-terminal half of the RLC crosslinks with ELC both at rest and in rigor without abolishing regulation.

Light chain movement does not require the presence of actin. This is in agreement with other observations that indicate that control is defined by the state of myosin. It has been shown some time ago that calcium activates the Mg-ATPase of molluscan myosin in the absence of actin (36). Single turnover studies have established that calcium increases the turnover rate of ATP from several minutes to a fraction of a second by accelerating the phosphate release step (37,38). These studies have also shown that the turnover rate in the absence of calcium is very low, myosin is sufficiently tightly controlled in vitro to account for the very

low ATP usage of living resting muscles. The effect of calcium on myosin has been directly observed by electron microscopy of negatively stained isolated thick filaments (39). In relaxing solutions the myosin heads are arranged in a regular helical pattern, but become disordered by calcium (40). The structure of the myosin filament is altered at similar calcium concentrations that are required for ATPase activation (41) and for tension generation (42). Thus calcium directly affects myosin structure and function.

The RLCs of the two myosin heads can also be cross-linked provided the cross-linker is attached to the N-terminal third of the RLC (43). The separation between the two myosin heads at a level corresponding to residue 50 of the RLC is therefore likely to be less than 10A. Electronmicroscopy of cross-linked myosin localized the position of the cross-linkers at the junction of the two myosin heads. S1 preparations obtained from cross-linked myosin by papain digestion produced dimers of the myosin heads connected at their narrow ends showing that cross-linking is very close to the head-rod junction (44). The close juxtaposition of the RLCs may account for the cooperative behaviour between the two myosin heads, in particular, that both of the heads need to bind calcium for ATPase activity (41).

It is significant that changes taking place at the junction of the two myosin heads influence interaction with actin, even though actin binds to myosin heads at a considerable distance (>100A) as indicated by electron microscopy(45). There is, therefore,a communication between widely separated regions of myosin. Such communication may be established by the interactions of myosin "domains" although a direct effect of the light chains on the actin binding site cannot be excluded (46). Communication between calcium and nucleotide binding sites has also been demonstrated following changes in intrinsic fluorescence (32). The tryptophan residue with the altered fluorescence in the presence of calcium is present on the regulatory peptide and is unresponsive to ATP. In contrast, S1 produced by papain in the absence of divalent cations and lacking

an about 3000 daltons fragment from its carboxyl end contains a tryptophan that is responsive to ATP but not to calcium. The understanding of structural transitions mediated over large distances is one of the central problems of the mechanism of subunit regulation of myosin.

PERSPECTIVES

This brief description of some of the findings on myosin linked regulation points out the multiple facets of light chain function; and demonstrates the usefulness of the sites that are available for modification at different positions on the light chains for the localization of functions such as divalent cation binding site, and light chain movement. The limitation of this approach is that there are, only few RLCs with a single thiol group at known position. Only three different RLCs have been used in the experiments mentioned. The RLCs of Mercenaria and gizzard muscles both confer regulation to desensitized scallop myosin and contain a single cysteine residue at positions 50 and 108 respectively. The behaviour of the carboxyl end of the RLC was tested with the aid of RLCs of vertebrate skeletal myosins that do not regulate and have two cysteine residues at position 129 and 157. In addition, other residues implicated in various LC functions cannot be chemically modified. Therefore, we have initiated a project in collaboration with Dr. Leslie A. Leinwand, Albert Einstein College of Medicine, utilizing site directed mutagenesis to study light chain functions.

Light chains of regulatory myosins are particularly suitable for mutational studies. They are relatively small simplifying the efforts involved in sequencing the cDNA clones. The light chains resist denaturation by detergents, urea, guanidine-HCl or trichloroacetic acid; it is therefore likely that once expressed, they can be recovered from E. coli in a functional state. Finally the properties of the mutant light chains can be tested on desensitized scallop myosins. Such an approach has been used with the RLCs of chicken fast

skeletal muscle myosin (47). The light chains were altered by oligo-nucleotide directed mutagenesis in domain-1. The divalent cation binding site of the mutant RLC was abolished, nevertheless, the RLC hybridized with desensitized scallop myosin. The mutant chicken RLCs were unable to inhibit ATPase activity the way wild type RLCs do when hybridized with desensitized scallop myosin (47). The RLCs of vertebrate striated myosins, however, do not confer calcium regulation when hybridized with scallop myosin. A detailed study of regulation by site directed mutagenesis requires the cloning of scallop light chains.

cDNA clones of myosin light chains from the scallop. Aequipecten irradians have been isolated from a λ gt11 expression library with the aid of affinity purified polyclonal antibodies directed against either the RLC or the ELC (48). From these clones the complete DNA sequences of the protein coding regions were obtained. The translated sequences of the ELC agree fully with the published sequence; the translated sequences of the RLC differ from the published sequences to obtained from the related species Pecten maximus (19) and Placopecten yessoensis (20); most of the differences are conservative replacements possibly due to species differences. The clones encoding the light chains hybridize with multiple transcripts although there is a single protein product for each light chains. Genomic DNA analysis shows that each protein is encoded by a single gene (48).

The properties of scallop myosin containing mutant RLCs and/or mutant ELCs should help to clarify the role of the light chains in myosin linked regulation. A successful analysis of a system by site directed mutation depends on the number of different properties that can be followed quantitatively. Light chains have a variety of well defined functions that are testable with relatively limited amounts of material. The following examples may be mentioned:

Binding of RLC to the heavy chains

Studies employing limited proteolysis of RLCs indicate that binding to the heavy chains requires an intact C-terminal regions. RLCs missing about 10 residues from the carboxyl and no longer bind to myosin. RLCs lacking about 50 residues from the amino terminus can still bind although with a reduced affinity (23,50). This is a somewhat surprising observation since scallop RLCs dissociate from the heavy chain in the absence of divalent cations that are bound by domain-1 near the N-terminal region (23). Mutants obtained by internal deletions and by controlled deletions of the ends may help to resolve this paradox. Similar studies can be applied to the ELC.

The role of divalent cations binding sites of the RLC

The divalent cation binding site of the RLC may be abolished by replacing an oxygen donating residue into a non-liganding one in domain-1 (e.g. Asp-28 into Leu) as it was previously done with chicken fast myosin RLC (47). Analysis of these mutants should help to clarify the role of this domain in binding to heavy chains, in specific calcium binding and in calcium sensitivity.

Contribution of the light chains to specific high affinity calcium binding

Although isolated RLCs do not bind calcium with a high affinity and specificity, the specific calcium binding site is not present in desensitized scallop myosin. It has been suggested that the ELC may be responsible for the specific calcium binding (5,10). This speculation is reinforced by the finding that in scallop ELC domain-3 contains an EF hand fully competent for divalent cation binding having in the -X-position a serine residue that in other species is methionine or isoleucine (49). Conversion of Ser-102 of scallop ELC into methionine by site directed mutagenesis and the effect of such amino acid replacement on calcium binding and calcium sensitivity could directly test the validity of this proposition.

Calcium sensitivity

RLCs from regulatory myosins restore calcium sensitivity in contrast to the ones that are obtained from non-regulatory ones. Deletions may pinpoint the regions of the RLCs that are responsible for these differences. The regions necessary for the inhibition of ATPase in the absence of calcium may also be identified with the aid of deletion mutants.

Analysis of light chain movement

Light chain movement was followed by cross-linking reagents that were covalently attached to thiols of various foreign light chains. The approach depends on the availability of light chains containing cysteine residues at different positions. The results were obtained with the aid of only three RLCs. Search for RLCs with thiols at additional positions was not successful (Titus, M. unpublished experiments). However, mutants of scallop light chains containing cysteine residues at any site desired can be readily constructed. This will allow for a more detailed description of light chain movement. It may allow to identify the interacting components in the "off" state. Reactions with the heavy chains or other muscle proteins may also be obtained.

Mapping of the light chains on myosin

Mutants containing thiols at different well defined positions may help to map the light chains on myosin by electron microscopy using the maleimidated biotin-avidine system (50) or gold clusters to visualize the thiol groups (52).

Difference between regulatory light chains

With the aid of mutants, one may be able to define which region or residue of RLC is necessary for regulation and determine why molluscan myosin RLCs function differently from vertebrate skeletal myosin RLCs.

We hope that the possibility offered by recombinant DNA techniques for changing residues at will at any position of

the light chain sequence will help to elucidate the molecular mechanism of subunit regulation of myosin.

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COMPUTER MODELING OF THE NEUROTOXIN BINDING SITE OF ACETYLCHOLINE RECEPTOR SPANNING RESIDUES 185 THROUGH 196*

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SUMMARY

A model of the complex between the acetylcholine receptor and the snake neurotoxin, cobratoxin, was built by molecular model building and energy optimization techniques. The experimentally identified functionally important residues of cobratoxin and the dodecapeptide corresponding to the residues 185-196 of acetylcholine receptor α subunit were used to build the model. Both cis and trans conformers of cyclic L-cystine portion of the dodecapeptide were examined. Binding residues independently identified on cobratoxin are shown to interact with the dodecapeptide AChR model.

INTRODUCTION

The nicotinic acetylcholine receptor(AChR) from electric fish organ is a well studied glycoprotein composed of four different polypeptide chains assembled into a transmembrane pentamer with the stoichiometry $\alpha_2\beta\gamma\delta$ (for reviews, see refs. 4,17, 28).

The cholinergic binding site for this receptor found in the extra cellular domain of the α-subunit. Based on affinity-labeling experiments a sulfhydryl group was suggested as being present in the proximity of the ACh binding site (13). Of the seven cysteines present in the α-subunit of the T. californica AChR, the hydrophilicity profile indicates that four (residues 128, 142, 192, 193) are involved in disulfide bridges (23). The importance of these cysteine

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residues was later confirmed by site directed mutagenesis which demonstrated that a mutation of any of the above mentioned cysteines to a serine eliminates completely the responsiveness of the AChR to ACh (18).

The primary ACh binding site was originally thought to involve the region including Cys 128 and Cys 142 for the following reasons (23). First, formation of disulfide bond between adjacent residues (192 and 193) was considered to be very unlikely. Second, the region containing Cys 128 and Cys 142 also includes the glycosylation Asn 141 site which is known to exist at the ACh binding site (23). This hypothesis has motivated various experimental studies(14, 18) as well as structural model studies (25, 26) for this region (Cys 128 - Cys 142) of the AChR molecule. Recently accumulated evidence from various studies including affinity labeling, toxin binding and antibody recognition experiments (5, 6, 11, 12, 14, 20, 21, 22, 32) now suggests that the region containing the cysteine residues 192 and 193, is involved in the primary ACh binding site. Some synthetic peptides have been used to attempt to identify the ACh primary binding site residues. For example, Wilson et al. (32) synthesized a 32mer corresponding to residues 173-204 of AChR, Mulac-Jericevic and Atassi (20) worked with a 17mer containing residues 182-198, and Neumann et al. (21, 22) examined a dodecamer containing residues 185-196. In all of these studies recognition of the fragments was found.

Although the secondary structure prediction of the AChR α subunit by the Fourier transform of the hydrophobicity values is available in the literature (8), the detailed 3-dimensional structural information needed for characterization of the binding site is not available. Snake venom toxins such as α-cobratoxin(CTX) and α-bungarotoxin(BTX) which are competitive inhibitors of ACh binding are peptides which can be used to model complex formed with the ACh binding site of AChR. The crystal structure of CTX (30) reveals that the molecule is folded into three loops which are cross linked by four disulfide bridges and have their long axes nearly parallel to each other. These loops form

a concave surface in the toxin molecule. This surface is believed to interact with the AChR, because functionally important residues are located in this surface (see Fig. 1).

In order to explore the structural features present in the primary ACh binding site, a computer modeling and an energy optimization study of the Neumann's dodecapeptide corresponding to Lys 185 to Thr 196 of AChR and the concave surface of CTX was made.

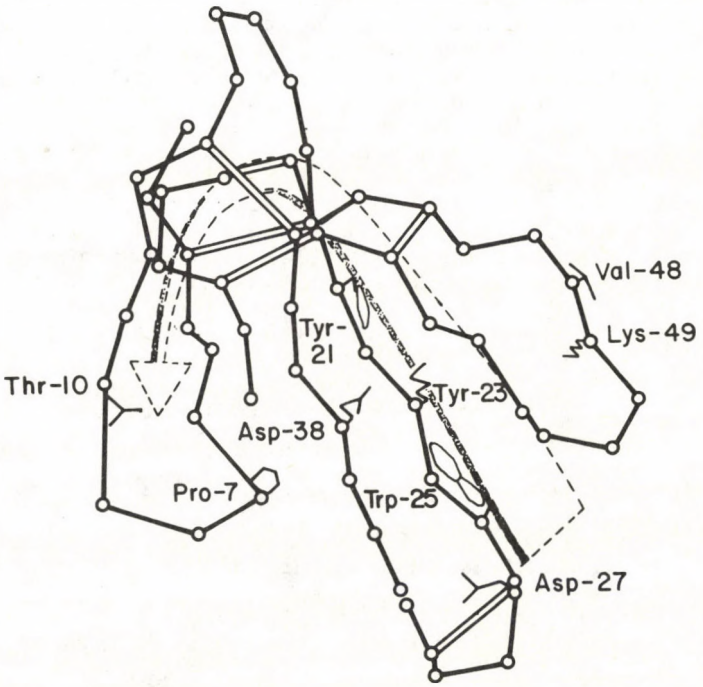

Fig. 1. Schematic diagram of the cobratoxin (CTX) and acetylcholine receptor complex model. The arrow depicts the location of the dodecapeptide where the tail corresponds to the N terminus and the head to the C terminus. Some of the functionally important residues of CTX are also shown in this figure.

METHODS

The initial geometry for Neumann's dodecapeptide, sequence KHWVYYTCCPDT, was made using the ECEPP program (1). The cis and trans conformers of the Cys192 and Cys193 peptide bond were built using GEOMOL, a model building program developed in this laboratory (24). A computer

graphics simulation program for the Evans and Sutherland PS300 (27) also developed in this laboratory, brought the 12mer into a possible geometric interaction configuration with the whole cobratoxin(CTX) crystal structure.

Once the 12mer -CTX complex was formed, the CTX portion was reduced to a minimum number of interacting residues. These consisted of three molecular fragments, i.e. residues 7 to 16 PDITSKDCPN, residues 21 to 27 YTKTWCD, and residues 38 to 50 DLGCAATCPTVKT. Lys23 and Lys49 of CTX where then reoriented so that Lys23 would be hydrogen bonded to the main chain of AChR, and that Lys49 interact with His186 of AChR. Stacking between Trp25 of CTX and Trp187 of AChR, and ionic contact between Asp27 of CTX and Lys185 of AChR were also introduced. All possible hydrogen bonds were included in the initial structure prior to energy optimization.

Energy optimization was done using the conjugate gradient method (9) and a potential function described by Weiner et al. (31). A distance dependent dielectric permitted the damping of long range interactions. The charged amino acid residues of the model such as histidine, aspartic acid, asparagine and lysine were in their ionized form. During the optimization process, the peptide backbone of CTX was fixed in cartesian space while side chain atoms of CTX as well as the whole dodecapeptide of AChR were allowed to move freely.

RESULTS

The model reported in this paper indicates that the dodecapeptide portion of AChR exists as a β strand followed by a turn generated by the Cys Cys Pro residues with the overall shape of a hook attached to the concave surface of the CTX, Fig. 1. Although the occurrence of a disulfide bond between adjacent cysteine residues is very rare in protein structures (29), X-ray structures of the synthetic cyclic L-cystine derivatives are reported in the literature (2, 10). A right handed hook was generated by use of the crystallographic coordinate of cyclic disulfide L-cysteinyl-L-cysteine (2) and a torsional parameter of a proline residue to simulate a turn structure, which pointed away from the upper part of the toxin concave surface. Manipulation of torsional angles including a rotation along psi angle of Cys 192 by 120 degree permitted reversal of the direction of the hook allowing it to interact with the CTX residues.

Although the available data suggested the existence of an unusual cis peptide bond in the cyclic L-cystine, the possibility of forming a trans conformer of the dodecapeptide

was also examined in this paper. The trans conformer was generated from cis conformer by torsional adjustments. Despite a significant difference in the local structure of cyclic disulfide region of the dodecapeptide, the overall shape, schematically depicted in Fig. 1 remained very similar.

The optimized structures of the CTX-AChR model complexes for the cis and the trans conformers are shown in Fig. 2 and 3 respectively (models C and T). The torsional angles involved in the cyclic L-cystine region of adjacent cysteine

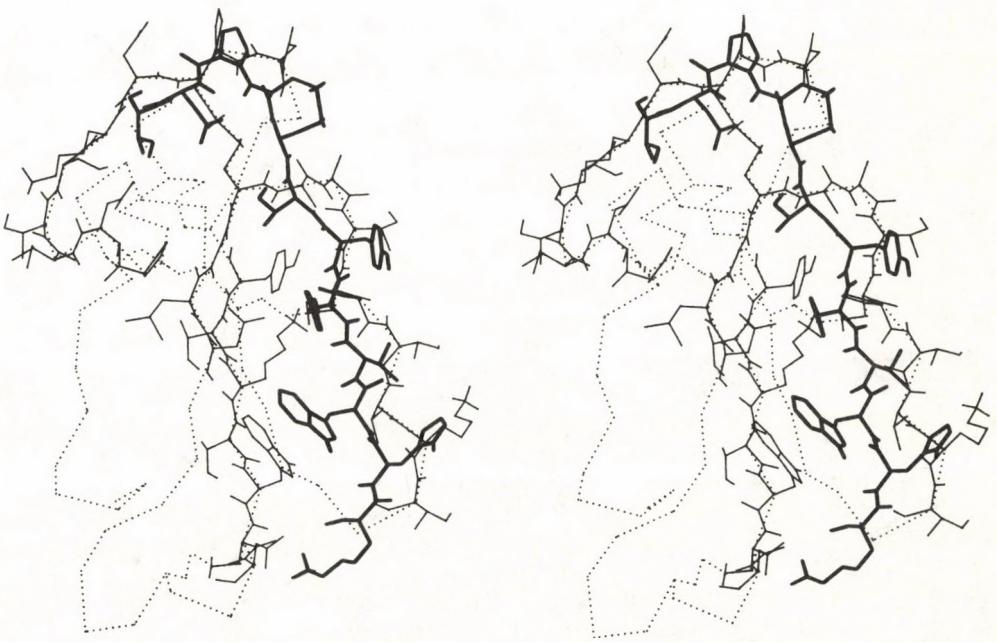

Fig. 2. Stereodiagram of the model complex between cobratoxin (CTX) and acetylcholine receptor dodecapeptide in its cis form. The dotted line depicts the cabon alpha trace of CTX backbone, the thin solid line is for the fragments 1 through 3 of CTX included in the optimization. The thick solid line is for dodecapeptide corresponding to residues 185-196 of acetylcholine receptor.

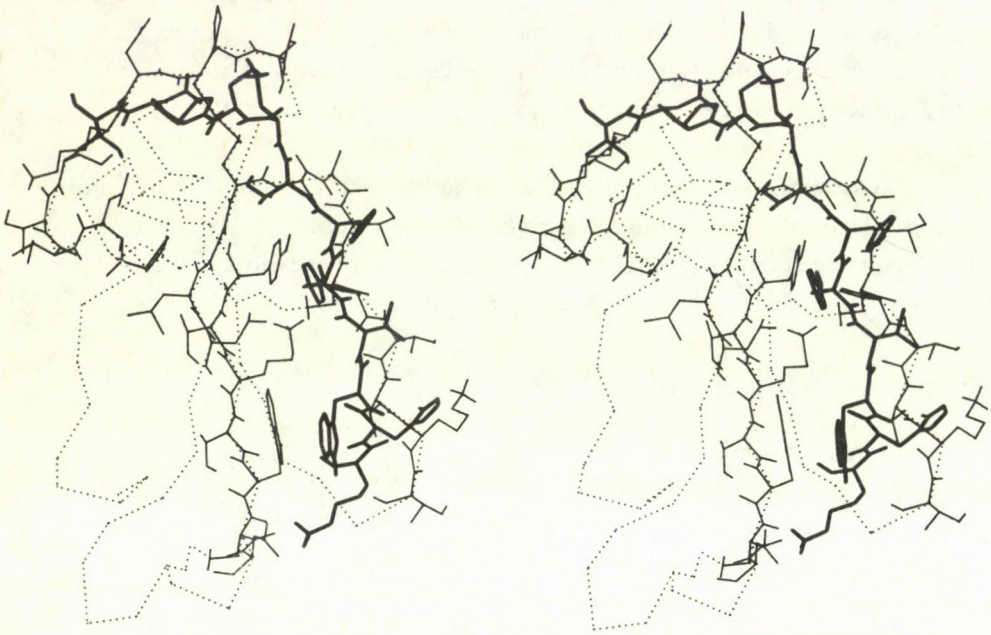

Fig. 3. Stereodiagram of the model complex between cobratoxin (CTX) and acetylcholine receptor dodecapeptide in its trans form (see Fig. 2 caption).

residues are given in Table 1, along with those found in x-ray crystal structures of synthetic cyclic L-cystine derivatives (2, 10) and with the values calculated for malformin A (19). Malformin A containing a D-cystine was included because it was the only available example of a trans conformer of a cyclic disulfide. Table 1 shows that the cyclic cystine induces a strain into peptide bond which shows up as a deviation from planarity for the ring. This is exaggerated in the model T, table 2, trans conformer which has a 32 degree deviation from the planarity. The theoretical calculations (3, 19) for the structure reported in Table 2 indicated that for each conformer there exist a right handed and a left handed disulfide bond with very similar stability. The results reported here are only for the left handed trans and the right hand cis forms. Also, most crystal

Table 1. Hydrogen bonding and ionic contact distances between cobratoxin (CTX) and acetylcholine receptor (AChR)

	CTX residue		AChR residue		Distances (A) trans model	cis model
Frag. 1	Asp (8)	O_δ	HC_γ	Thr (196)	2.65	2.63
	Ser (11)	$O_\gamma H$	O	Asp (195)	2.68	
Frag. 2	Tyr (21)	O_η	HN	Tyr (190)	2.94	3.12
	Tyr (21)	$O_\eta H$	O	Tyr (190)	2.67	2.70
	Lys (23)	$N_\xi H$	O	Val (188)	2.64	2.78
	Asp (27)	$O_\delta{}^1$	$H_a N_\xi$	Lys (185)	2.54	2.54
	Asp (27)	$O_\delta{}^1$	H N_ξ	Lys (185)	2.54	2.54
	Asp (27)	$O_\delta{}^2$	H N_ξ	Lys (185)	2.57	2.57
Frag. 3	Cys (41)	NH	O_γ	Thr (191)	2.83	2.71
	Cys (41)	O	HO_γ	Thr (191)	2.74	2.74
	Cys (41)	O	HN^γ	Cys (192)	3.03	
	Thr (44)	O	HO_η	Tyr (190)	2.75	
	Lys (49)	$N_\xi H$	N_ε	His (186)	2.87	2.78

structures for disulfide bridge have a dihedral angle xs, defined by Cβ-S-S-C β, in the vicinity of ± 90 degrees. The corresponding angle in models T and C is around 60 degrees. This may be due to the placement of sulfur lone pair electrons required by the energy parameters (31) used in this study. This may have played a role in the elongation of the S-S bond from 2.03 A as found in crystals to approximately 2.14 A reported here.

The hydrogen bonding and ionic contact distances for the complexes shown in figures 2 and 3 are reported in Table 2. It can be seen that the AChR models have hydrogen bond distances and ionic contacts near the normal values. The stacking distance between the two Trp's in both complexes lies near 3.5 angstroms. An additional hydrophobic contact between Val 189 (AChR) and Thr 47 (CTX) give seven out of 12 residues of the cis form of the dodecapeptide interacting with the CTX.

Table 2. Dihedral angles involved in the disulfide bridge by the adjacent cysteines

Angle[g]	Crystal		Theoretical calculations[c]							
			CysCys[d]		Malformin A[e]				Models[f]	
	I[a]	II[b]	L	R	L_1	R_1	L_2	R_2	T	C
ψ_i	156	153	132	150	-142	-142	68	56	-81	-163
ω_i	-7	10	-12	14	-6	10	165	165	-148	3
ϕ_j	-129	-136	-150	-138	125	123	154	139	-151	-123
X_{1i}	-154	-164	177	-165	155	-151	-151	-79	-173	67
X_{2i}	-81	-77	52	-79	79	-72	96	-79	-46	66
X_s	94	95	-98	100	-92	91	-94	110	-57	44
X_{2j}	-48	-49	75	-51	64	81	-31	31	92	-61
X_{1j}	-54	-59	-74	-57	38	-59	79	-64	-55	-45

a) Cyclic disulfide L-cysteinyl-L-cysteine from Ref. 2.
b) Tert-butyloxycarbonyl-L-cysteinyl-cysteine disulfide methyl ester from Ref. 10.
c) Right handed (R) and left handed (L) disulfide bond.
d) Theoretical calculation of cyclic disulfide cysteinyl-cysteine from Ref. 3.
e) Theoretical calculation of Malformin A. from Ref. 19.
f) Trans and cis conformers studied in this paper.
g) Torsional angle definition can be found in IUPAC-IUB nomenclature Biochem. 9, 3471-3479 (1970).

DISCUSSION

Functional transitions of the acetylcholine receptor involved in the regulation of ionic channel are induced by binding of acetylcholine to the receptor binding site. Identification of a dodecapeptide in the primary sequence in the acetylcholine receptor as the primary binding site has been reported (21, 22). Also, structure function studies on curarimimetic snake toxins have indentified the important residues involved in binding of these toxins to the acetylcholine receptor (7, 15).

The primary objective of the present paper was to develop a 3-D model of the interaction complex between the Neumann dodecapeptide representing the acetylcholine receptor binding site and cobratoxin. These models are described in Fig. 2 and 3 and Tables 1 and 2.

Table 3. Functionally important residues of cobratoxin

Residue	Sequence[a]	Structure Activity[b]	Sequence Analysis[c]	Model[d]
S/T	9 (6)		66	-
T	13 (10)		33	
S/T	14 (11)		66	D (195) (trans)
Y	25 (21)	F5		Y (190)
K	27 (23)	F5	66	V (188)
T	28 (24)		33	
W	29 (25)	F2	100	W (187)
D	31 (27)	F1	66	K (185)
R	37 (33)	F1	100	-
G	38 (34)		100	-
R/I/V	40 (36)	F2		-
D/E	42 (38)	F5	66	
P	50 (46)	F5		
V	52 (48)	F4	66	
K/R	53 (49)	F3	66	H (186)
G	56 (51)		66	-
V/I	57 (52)	F2		-
I/V/L	59 (54)	F4		-

a) The residue numbers are given in the Karlsson system and the corresponding actual sequence numbers are shown in parantheses.
b) Five groups (F1-F5) of functionally important residues identified by structure activity study (15).
c) Functionally important residues identified by sequence analysis study (7). The percentages of occurrence in neurotoxin sequences, 100 for invarient, 66 for above 66% and 33 for between 33% and 66%, are given.
d) AChR residues which are interacting with the specified CTX residues obtained by this study. The symbol - indicates the residues not included in the model.

According to these models, the dodecapeptide forms a β strand followed by the disulfide bridge between adjacent cysteines and a proline residue as the structural element responsible for locking in the turn. The results reported here show that the turn region can be realized either in the cis or trans conformation of the cyclic peptide region of the disulfide bridge. Both can form a complex with the cobratoxin stabilized by hydrogen bonds and stacking interactions. It was observed that the interaction is 12 kcal/mol stronger for the trans than the cis form. This is supported also by

the hydrogen bonding geometry. Nevertheless, two unfavorable factors in trans form, the intrastrand electrostatic interaction and the torsional term, favor the overall stability of the cis form. This indicates that there is an important balance between intermolecular interaction between CTX and AChR on the one hand and the conformational energy of AChR itself on the other. The model used here involved a selection of important residues and a fixed backbone of the toxin. In order to decide if the cis or trans form complex is more stable a larger scale model would be needed. This model would include a flexible backbone as well as some of the additional residues which may play a role in stabilizing the complex (for example, Val 52) during the optimization.

Other discussions aside, it is more important to compare the modeling data to the results obtained by structure-function studies (15) and sequence analysis of neurotoxins (7). Low (15) has identified five groups of residues in the snake toxins which are involved in binding to AChR. Functionally important residues are also indentified by searching for the residues found in neurotoxins but not in cardiotoxins (7). Both sets of residues are shown in Table 3 where the interacting residues in our model are also identified. From this table it can be observed that four of the five experimentally identified functionally important residue groups of CTX interacting with AChR are represented by at least one residue in our model. Another interesting observation is that only one of the Arg and Asp pair of toxin is involved in the interaction in our model. This Arg and Asp pair located at the tip of middle loop of toxin has been considered to mimic ACh molecule. The absence of Arg in our models raises the possibility that the actual binding site may span more than 12 residues. Although it is not clear how many residues are involved in the binding site of AChR, the models clearly indicate the existence of a disulfide bond formed by Cys 192 and Cys 193 near the ACh binding site in agreement with experiments (12). This may be an indication that the vicinal disulfide may play an important role in the activation of

receptor molecules by the agonist similar to the role played by the disulfide bridge in β-adrenergic receptors (16).

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ATROPINE IS AN INHIBITOR OF THE CHEMILUMINESCENCE INDUCED BY PLATELET-ACTIVATING FACTOR IN HUMAN NEUTROPHILS

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SUMMARY

Chemiluminescence provoked by platelet-activating factor can be dose-dependently inhibited by atropine. This effect of atropine is rather due to its ion channel blocking capability (at the higher doses than 10^{-5} M) than to its action on the acethylcholine receptors. The differences in the roles of platelet-activating factor and acethylcholine in the activation of phagocytes are discussed.

INTRODUCTION

The platelet-activating factor is now recognized as a phospholipid mediator of inflammation, since it is released from the most stimulated pro-inflammatory cells (1, 2, 3). PAF is found to increase directly the superoxide anion generation (4) and the C3b and Fc receptor-mediated chemiluminescence of human neutrophils (5). It also has been demonstrated that - beside platelets (6) - the human PMN cells possess a specific binding site for PAF (7).

We have found earlier that the CL induced by opsonized zymosan in human neutrophils can be inhibited by atropine (8). Acetylcholine and carbamylcholine, the stable form of acetylcholine, can stimulate this process (8). Since PAF and acetylcholine have two chemical groups in common, acetyl and choline, in this work we tested the effects of atropine,

Abbreviations: PAF, platelet-activating factor; CL, chemiluminescence; HBSS, Hank's balanced salt solution

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the blocking agent of the acetylcholine receptor, on the CL induced by PAF in human neutrophils. We found that atropine can dose-dependently inhibit the chemiluminescence provoked by PAF.

1-0-hexandecyl-2-acetyl-sn-glyceryl-3-phosphoryl-choline, PAF (Bachem, Switzerland) was freshly dissolved in Hank's balanced salt solution completed with 0.2% bovine serum albumin (Sigma, USA). Acetylcholine chloride, carbamylcholine chloride, atropine were purchased from Sigma. The reagents were freshly dissolved in HBSS before the experiments.

Neutrophil suspensions containing 95-98% neutrophils were prepared from heparinized venous blood obtained from healthy adult donors. After employing Ficoll (Pharmacia, Sweden) - Uromiro (Branco Ind. Chim., Italy) gradient centrifugation, the pellet was sedimented by dextran (Macrodex, 70,000 m.w., Pharmacia) and the hypotonic lysis of red cells took place in destillated water for 20 sec. The neutrophils were suspended in HBSS (9).

10^6 neutrophils were preincubated at 37°C for 5 min in the presence of 10^{-4} M of luminol (Sigma) in 1 ml final volume with different amounts of atropine in plastic tubes, then the suspension was poured into the cuvette of the luminometer (LKB, type 1250, Wallac, Finnland). The chemiluminescence inducing effects of 10^{-6} M of PAF was measured on the neutrophils as soon as it was injected into the suspensions, at 37°C under permanent magnetic stirring. The emission of photons per second was automatically recorded and finally expressed in cpm.

PAF in 10^{-6} M concentration could induce CL directly in the human neutrophils (5) whereas lyso-PAF, acetylcholine and carbamylcholine were ineffective even at higher concentrations than 10^{-4} M.

Preincubation of human neutrophils with different amounts of atropine (Fig. 1) resulted in a marked concentration-dependent decrease in the PAF induced CL of the PMN cells.

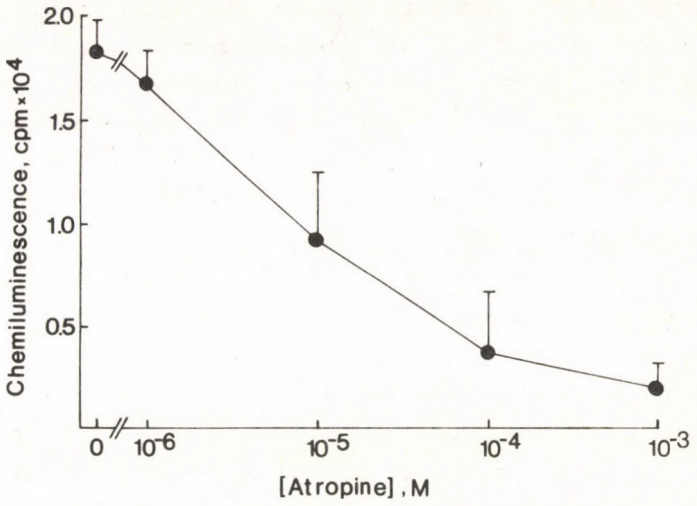

Fig. 1. Effect of atropine on the chemiluminescence induced by 10^{-6} M PAF in human neutrophils. 10^{6} PMN cells were preincubated with various concentrations of atropine for 5 min at 37°C, then the CL was measured. The number of photons emitted after the injection of PAF was expressed in cpm. The figure represents the means and ± SD of 5 experiments.

Atropine was non-toxic in the concentrations causing the inhibition in the CL of neutrophils until the 7th min of incubation as it was demonstrated by trypan-blue exclusion test, at the time of CL measurements.

The human phagocytic cells contain cell membrane receptors for both acetylcholine (10) and PAF (7). Although, acetylcholine and PAF have chemical structures in common, it was only PAF which could directly induce CL in the human neutrophils. This finding suggests that the acetylcholine and the PAF receptors of the human PMN cells can not be identical. At the same time, our data concerning the inhibitory effect of atropine, which is a blocking agent of the muscarinic acetylcholine receptor, need additional explanations, too. From the results the following conclusions can be drawn: 1. the two different receptors are localized close to each other, 2. they may be structurally similar, and

3. atropine may have some other membrane effects, as well. We favour the third variant, because it is known that atropine at higher concentrations (10^{-5} M) can block the open Na^+, K^+ and Ca^{2+} ion channels of the cell membrane (11, 12). Besides, atropine can also act on the adrenergic receptors (13). In the mechanism of provoking CL, the increased influx of Na^+ and Ca^{2+} ions into the phagocytosing cells plays a crucial role (14). Therefore, the agents blocking the membrane channels of these ions can decrease the CL of the cells (15), like atropine in our experiments. It can be stressed that the function of the PAF receptor on the surface of various cell types can be partly similar to the acetylcholine receptor, namely, being a gate channel for the ions Na^+ and Ca^{2+} (11, 16). However, the actions of PAF seem to be more complex than an ionophore-like mechanism (17).

The role of PAF in the inflammation can be attributed to two properties: 1. direct activation of the resting phagocytic cells and 2. stimulation of the metabolic, phagocytosing and killing activities of cells producing further quantities of PAF (5).

The importance of acetylcholine and acetylcholine receptor in the regulation of phagocytosis also can not be completely ruled out. Apparently, the human neutrophils can not synthesize acetylcholine during phagocytosis (8), but they can produce PAF (1, 2, 3). According to our data, acetylcholine does not activate directly the neutrophils in resting state, however, in the state of phagocytosis, it does (8).

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BIVALENT CATION AND ATP REQUIREMENTS OF ENDONUCLEASES FROM RAT LIVER NUCLEI

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SUMMARY

The distribution of different types of DNase in rat liver nuclei was determined after a purification procedure involving ion exchange chromatography and gel filtration. As major enzymes Ca^{2+}, Mg^{2+}-dependent endonuclease, Mn^{2+}-dependent endonuclease and an acid endonuclease were identified, sharing 60, 20 and 10 % of the total activity, respectively. Mn^{2+}-dependent endonuclease is a novel enzyme with a molecular mass of 30 + 5 kilodaltons. The synergistic effect of Ca^{2+} and Mg^{2+} ions for the Mn^{2+}-dependent enzyme was lower by an order of magnitude than that of the Ca^{2+} and Mg^{2+}-dependent endonuclease. The Ca^{2+} and Mg^{2+}-dependent nuclease activity represents a heterogeneous population of enzymes. One of the cation dependent enzymes (M_r 25 $\pm$ 5 kD) is stimulated by ATP the ATP optimum being 0.1 mM and the Mg^{2+} requirement 1 mM. The ATP-dependent endonuclease belongs to the minor endonucleases separable from the major ones.

INTRODUCTION

Eukaryotic endonucleases associated with the chromatin structure are classified by their dependency on bivalent cations. Ca^{2+} and Mg^{2+}-dependent endonucleases (Ishida et al. 1977; Yoshihara et al. 1974; Khodarev et al. 1979; Nakamura et al. 1981; Nikonova et al. 1982; Bubnov et al. 1987), Mg^{2+}-dependent endonucleases (Yoshihara et al. 1974; Nikonova et al. 1982; Machray, Bonner, 1981; Tanigawa, Shimoyama, 1983) were described. There exist also acid endonucleases independent of cations (Nikonova et al. 1982;

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Slor, Lev, 1971). We have described earlier a new endonuclease in rat liver, hydrolyzing double stranded DNA in the presence of Mn^{2+} (Bubnov et al. 1987). Mn^{2+}-dependent endodeoxyribonuclease from rat liver nuclei causes a more dispersive cleavage pattern than that of the Ca^{2+}, Mg^{2+}-dependent endonuclease. The Mn^{2+}-dependent enzyme appears to be more specific to repeated sequences of DNA. So far there are only few endodeoxyribonucleases which are bivalent cation-dependent. It is not known yet whether or not the Mg^{2+}-dependency is a specific property and satisfactory criterion for the classification of endonucleases.

Another sign that might be used for classification and indicate the role of endonucleases or related enzymes is the ATP requirement. Such examples are the prokaryotic rec BC enzyme of E.coli, catalyzing homologous recombination (Taylor et al. 1985), type II topoisomerases (Champoux, 1978) which are involved in replication and introduce double-strand breaks in DNA in the presence of ATP (Tewey et al. 1984) and the purified receptor for epidermal growth factor isolated from membrane vesicles of A-431 cells, which is able to nick supercoiled DNA in an ATP-stimulated manner (Mroczkonski et al. 1984).

Earlier papers have primarily dealt with the isolation and characterization of individual endonucleases (Ishida et al. 1977; Yoshihara et al. 1974; Nakamura et al. 1981; Tanigawa, Shimoyama, 1983), less attention was paid to groups of enzymes (Khodarev et al. 1979; Nikonova et al. 1982). In this paper we give an account of the relative activities of several nuclear endonucleases in rat liver. For this purpose Ca^{2+}, Mg^{2+}-dependent, Mn^{2+}-dependent and acid endonucleases were isolated. In addition to these endonucleases, ATP-dependent nuclease, ATPase and topoisomerase activities were traced.

MATERIALS AND METHODS

Buffer TCS-A: 50 mM Tris-HCl (pH 7.9), 10 mM $CaCl_2$, 5 mM mercaptoethanol, 0.25 M sucrose. Buffer TCS-B: 50 mM Tris-HCl (pH 7.9), 10 mM $CaCl_2$, 5 mM mercaptoethanol, 2 M sucrose. Lysis buffer: 50 mM Tris-HCl (pH 7.5), 2 M NaCl,

50 mM EDTA, 5mM mercaptoethanol, 20 % (v/v) glycerol. Storage buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM dithiotreitol, 50 % (v/v) glycerol. Buffer A: 20 mM sodium acetate (pH 5.7), 0.1 mM EDTA, 5 mM mercaptoethanol, 10 % (v/v) glycerol. Buffer B: buffer A plus 1.5 M NaCl. Buffer C: 40 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 1 mM EDTA, 5 mM mercaptoethanol, 10 % (v/v) glycerol.

[γ-^{32}P]-ATP (200 Ci/mol) was prepared according to Post and Sen (1967).

Rat liver DNA was isolated by the method of Marmur (1961) with some modifications including RNase A and proteinase K digestion as described earlier (Bubnov et al. 1987; Bubnov et al. 1985). More than 98 % of DNA appeared as a single, undegraded band in 0.8 % agarose gel. Plasmid pBR 322 DNA was isolated as described by Maniatis et al. (1982). It consisted of 90 % supercoiled DNA and 10 % nicked DNA. T7 DNA was prepared according to Richardson (1966). Heat denaturation of DNA was carried out at 100 °C for 10 min followed by rapid chilling in ice.

Isolation of nuclei

Nuclei were extracted from nonimbred male rats (150-180 g) fed ad libitum. Livers from 20 rats were isolated as described (Bubnov et al. 1987; Bubnov et al. 1985). All operations were carried out at 2-4 °C.

Tissue was homogenized in TCS-A buffer containing 1/100 volume of 100 mM phenylmethylsulfonyl fluoride (PMSF) and filtered through steril gauze. Nuclei were harvested by centrifugation at 1500 g for 10 min. The pellet was suspended in buffer TCS-B and centrifuged through a gradient containing 2 M sucrose at 70 000 g for 90 min in a Beckman SW 27 rotor. Sedimented nuclei were washed twice in TCS-A buffer by centrifugation at 1000 g for 5 min. Nuclei were resuspended in the same buffer to a final concentration of 2 mg/ml DNA.

Nuclear extract

Nuclei were suspended in TCS-A buffer containing 1 mM PMSF, an inhibitor of proteinolysis, and 10 mM $CaCl_2$ to prevent endonuclease activity. Extraction of enzymes was carried out by adding an equal volume of lysis buffer dropwise by constant stirring to the nuclei suspension. After mixing for another 20 min the suspension was centrifuged at 30 000 g for 30 min. Freshly prepared 10 % streptomycin sulphate solution was slowly added to the supernatant by constant stirring over 20 min. The volume of the streptomycin sulphate was 1/20 that of the supernatant. The suspension was centrifuged at 50 000 g for 40 min and the pellet containing nucleic acids was discarded. The supernatant was concentrated by ultrafiltration through Amicon YM 10 filter. This fraction was dialyzed against storage buffer and stored at -20 °C for over 1 month without considerable loss of enzyme activity.

Assay of endonuclease activity

Acid endonuclease activity was determined in a reaction mixture (20 µl) containing 2 µg rat liver DNA, 10 mM sodium acetate buffer (pH 5.0), 0.5 mM dithiotreitol, 25 µg/ml bovine serum albumin, 1 mM EDTA and enzyme.

Bivalent cation dependent endonuclease activity was measured at 37°C for 1 hour in a reaction mixture (20 µl) containing 2 µg rat liver DNA, 10 mM Tris-HCl (pH 7.7), 0.5 mM dithiotreitol, 25 µg/ml bovine serum albumin, bivalent cations and enzyme.

Endonuclease activity was measured in all probes and chromatographic fractions. To distinguish among the ion dependency, nuclease activities of each probe were assayed under four different conditions: 1. Ca^{2+} + Mg^{2+}, pH 7.4; 2. Mg^{2+} at pH 7.4; 3. Mn^{2+} at pH 7.4; 4. EDTA at pH 5.0.

ATP-stimulated endonuclease activity was carried out under the same conditions in the absence or in the presence of 1 mM ATP, using 5 mM $MgCl_2$ concentration. Reactions were stopped by the addition of 5 µl 1 % sodiumdodecyl sulfate, 0.5 M EDTA, 0.25 % bromphenol blue, 50 % glycerol (v/v). Digested DNA was separated by electrophoresis in 0.8 % agarose gel. DNA was stained with 0.5 µg/ml ethidium bromide and gels were photographed and scanned in a Joice-Loebl densitometer as described (Bubnov et al. 1987; Bubnov et al. 1985). One unit of endonuclease represents that amount of enzyme which causes 50 % of rat liver DNA (1 µg) to migrate from its starting position in agarose gel in 1 hour under the conditions mentioned above.

DNA topoisomerase assay

Twenty microliters of the reaction mixture contained 1 µg supercoiled pBR 322 DNA, 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 50 mM NaCl, 1 mM EDTA, 25 µg/ml bovine serum albumin and enzyme. For the determination of type II topoisomerase activity probes containing 1 mM ATP were run simultaneously. Incubation was at 37 °C for 30 min. The reaction was stopped by the addition of 2.5 µl 1 % SDS, 0.1 M EDTA plus 2.5 µl of 500 µg/ml proteinase K. Proteinase K digestion was for 30 min at 37 °C. One unit of topoisomerase relaxes 0.5 µg supercoiled DNA in 30 min under the conditions mentioned.

DNA-dependent ATPase assay

ATPase activity was assayed by the measurement of ^{32}P liberated from [γ-^{32}P]ATP as previously described (Bánfalvi et al. 1981). One unit of ATPase is capable of hydrolyzing 1 nmol of ATP to inorganic phosphate and ADP in 20 min at 37°C.

Determination of proteins

Protein was determined by the method of Badford (1976), using crystalline bovine serum albumin as standard.

RESULTS

Endonucleases in extracts of rat liver nuclei

The basic level of endonuclease activity was determined in nuclear extracts. A summary of the most significant endonuclease activities of isolated enzymes is shown in Table 1.

Table 1. Enzyme activities in a nuclear extract

Enzyme	Specific activity, units/mg protein
Endonuclease	
+ Ca^{2+}, + Mg^{2+}	2.7×10^2
+ Mg^{2+}	0.4×10^2
+ Mn^{2+}	0.8×10^2
+ EDTA (pH 5.0)	0.7×10^2
Topoisomerase	
- ATP	2.0×10^6
+ ATP	1.5×10^6
ATPase	
- DNA	1.0×10^2
+ denatured T7 DNA	1.3×10^2

A nuclear extract containing 1 M NaCl was dialyzed against storage buffer. Concentrations of cofactors and measurements of enzyme activities are described in "Materials and Methods".

The synergistic effect of Ca^{2+} and Mg^{2+} is expressed as the ratio of Ca^{2+} + Mg^{2+}/Mg^{2+}-dependent nuclease activities. The synergistic effect in nuclear extracts was about 7-fold. Endonuclease activities were higher in the presence of Mn^{2+} or EDTA at pH 5.0 than in the presence of Ca^{2+} and Mg^{2+}. The activity of topoisomerase was by several orders of magnitude higher than that of endonuclease. ATPase activity

was significant in nuclear extracts. DNA-dependent ATPase activity was undetectable owing to incomplete removal of nucleic acids from the cell lysates.

Separation of enzymes

The nuclear extract was dialyzed against buffer A and adsorbed to phosphocellulose column (1.6 x 10 cm) equilibrated with the same buffer. The column was washed with buffer A and eluted with a linear salt gradient of 0 - 1.5 M NaCl in buffer A. Five regions (I-V) with endonuclease activity were distinguished by this method, (Fig. 1a, b). The detection of endonucleases revealed the presence of an acid endonuclease (peak I) which eluted at low ionic strength. The major activity of Ca^{2+}, Mg^{2+}-dependent endonuclease was found in peaks III, IV and V eluting at 0.5, 0.6 and 0.8 M NaCl concentrations, respectively. Endonucleases measured in peaks III, IV and V represented a heterogeneous population. The synergistic effect of Ca^{2+}, Mg^{2+}-dependent nuclease activity was the highest in the last two groups. Peak II was the highest of the three groups of enzyme. It was also heterogeneous but, contrary to III and IV, this population of endonucleases was Mg^{2+}-dependent.

ATPase activity in the same chromatographic fractions was detected simultaneously (Fig. 1d). Significant ATPase was detectable in peaks II and V and a low level of ATPase was traced in peaks I, III and IV. ATP-dependent DNase was undetectable in these chromatographic fractions.

In addition to endonuclease and ATPase, peak V contained topoisomerase and exonuclease activities.

Molecular mass determination

Groups of nucleases separated by phosphocellulose chromatography were gel-filtered using Toyopearl HW 60F (2.4 x 40 cm). In the emerging fractions bivalent cation and ATP requirements were determined. Groups III and IV

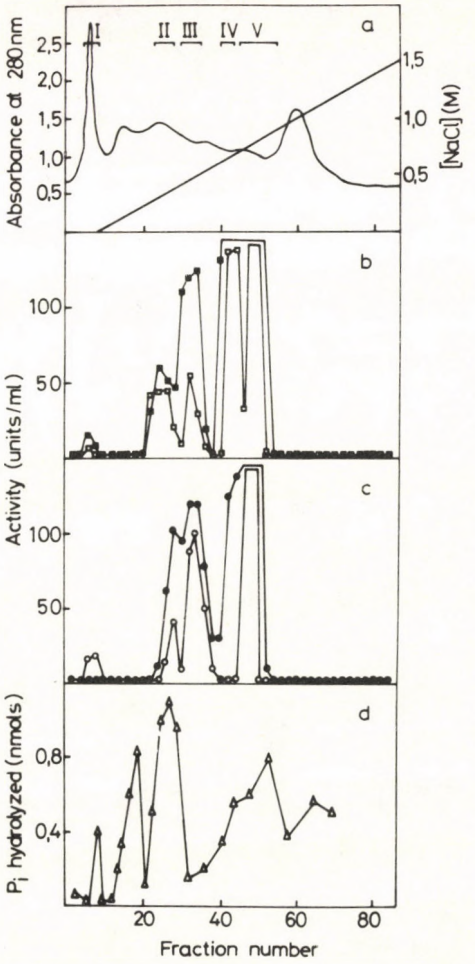

Fig. 1. Phosphocellulose chromatography of endonucleases and ATPases from rat liver nuclei. Nuclear extract was loaded on a column and eluted with a linear gradient (200 ml total volume) from 0 to 1.5 M NaCl. Absorbances of chromatographic fractions (2 ml) each were detected at 280 nm (a). Endonuclease activity was measured as described in "Methods" in the presence of Ca^{2+} + Mg^{2+} (□——□), Mg^{2+} (■——■) (b), Mn^{2+} (●——●) at pH 7.4 and under acidic conditions (pH 5.0) in the presence of EDTA (o——o) (c). ATPase activity (Δ——Δ) was determined by measuring liberated inorganic phosphate according to "Methods" in the presence of denatured T7 DNA as cofactor (d). Roman numerals designate pools of chromatographic fractions which were used for the following gel filtration.

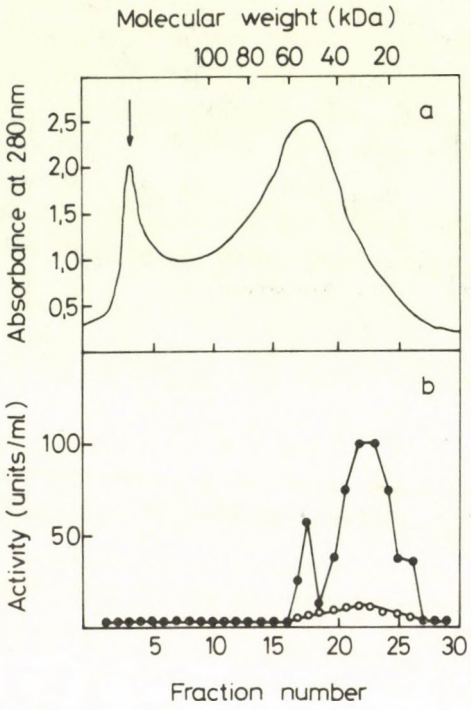

Fig. 2. Gel filtration of Mn^{2+}-dependent endonuclease, pool II. Pool II of phosphocellulose chromatography was concentrated to a volume of 4 ml using dialysis against polyethylenglycol 20 000 and loaded onto Toyopearl HW 60F column and eluted using buffer C and an elution speed of 1 ml/min. As molecular mass strandards human haemoglobin (67 kD), bovine serum albumin fraction V (62 kD), pancreatic DNase I (31 kD) and egg lysozyme (14 kD) were used. Blue dextran was added to each probe before loading. a. Absorbance at 280 nm of chromatographic fractions. Arrow indicates the mobility of blue dextran. b. Mn^{2+}-dependent nuclease (● —— ●) and acid endonuclease (o —— o) activities.

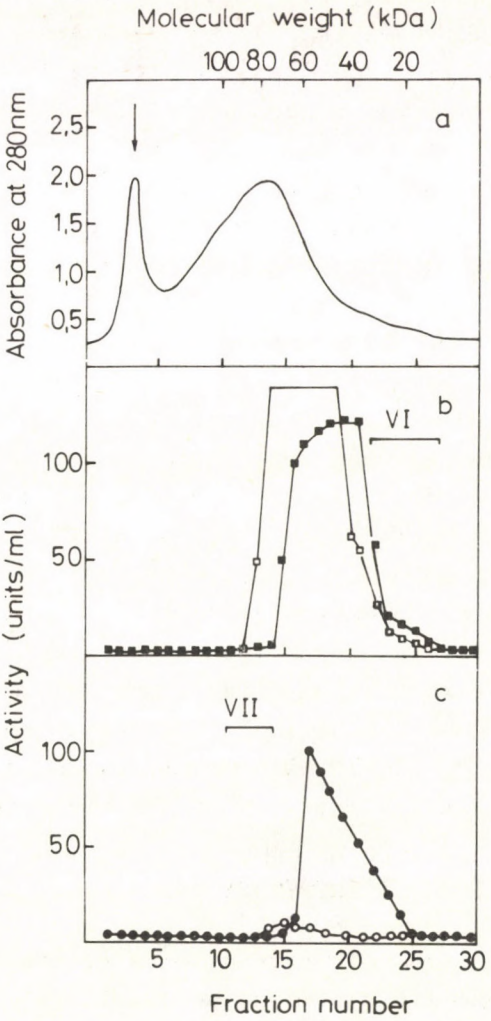

Fig. 3. Gel filtration of Ca^{2+}, Mg^{2+}-dependent endonucleases, pool V. Pool V of phosphocellulose chromatography was used for gel filtration under conditions described in Fig. 2. a. Profile of absorbtion at 280 nm. Pool VII represents fractions containing double stranded DNA binding proteins and DNA topoisomerase activity. Endonuclease activity in the presence of b. Mg^{2+} (■——■), Mg^{2+} + ATP (■---■), Ca^{2+} + Mg^{2+} (□——□) and c. Mn^{2+} (● —— ●) at pH 7.4 and in the presence of EDTA at pH 5.0 (o —— o).

consisted of a heterogeneous polulation of endonucleases with a molecular mass of 30-60 kilodalton (results not shown). The Ca^{2+}/Mg^{2+} synergistic effect was the highest in these groups. We consider these two groups as Ca^{2+}, Mg^{2+}-dependent endonucleases.

Group II represented a population of Mn^{2+}-dependent enzymes. Based on its chromatographic profile on Toyopearl column the average molecular mass of the material peak II was calculated tc be 30 $\pm$ 5 kilodaltons (Fig. 2). The mobility of the minor peak in Fig. 2 corresponded to a molecular mass of about 55 $\pm$ 5 kilodaltons. There was no Ca^{2+}, Mg^{2+}-, or Mg^{2+}-dependent nuclease activity in these chromatographic fractions. Group V was the most heterogeneous population of enzymes (Fig. 3). It consisted of Ca^{2+}, Mg^{2+}-dependent endonucleases (Fig. 3b). Fractions 11 - 14 (M_r 70 - 100 kilodaltons) contained a double-stranded DNA-binding protein which was detected in the presence of Mn^{2+} ions using rat liver DNA as substrate. DNA bound to this protein did not enter 0.8 % agarose gels. This protein turned out to be a mixture of topoisomerases. ATP-stimulated endonuclease was present in fractions 22 - 26 (M_r 25 $\pm$ 5 kD). These fractions were used for further analysis of cation and ATP dependency.

Requirements of ATP-stimulated endonuclease

Data on the cation and ATP-requirements of the ATP-stimulated endonuclease are shown in Table 2. This enzyme appeared to be one of the Ca^{2+}, Mg^{2+}-dependent endonucleases of low molecular mass and low Ca^{2+}/Mg^{2+} synergism. Optimal ATP concentration in the presence of 1 mM $MgCl_2$ was 0.1 mM.

Requirements of major endonucleases

The three major endonucleases localized in rat liver nuclei are Ca^{2+}, Mg^{2+}-dependent endonuclease, Mn^{2+}-dependent endonuclease and acid endonuclease. The activities of these enzymes under different conditions are summarized in Table 3.

Table 2. Bivalent cation and ATP-requirements of ATP-stimulated endonuclease

Reaction conditions	Specific activity, $x10^3$ units/mg protein
2 mM $CaCl_2$ + 5 mM $MgCl_2$	2.9
2 mM $MnCl_2$	1.0
1 mM EDTA (pH 5.0)	1.0
0 mM $MgCl_2$	1.0
0 mM $MgCl_2$ + 1 mM ATP	0
0.01 mM $MgCl_2$	0.5
0.01 mM $MgCl_2$ + 1 mM ATP	0
0.1 mM $MgCl_2$	0
0.1 mM $MgCl_2$ + 1 mM ATP	0
1 mM $MgCl_2$	5.0
1 mM $MgCl_2$ + 0.01 mM ATP	15.0
1 mM $MgCl_2$ + 0.1 mM ATP	17.0
1 mM $MgCl_2$ + 1 mM ATP	15.0
1 mM $MgCl_2$ + 2 mM ATP	10.0
1 mM $MgCl_2$ + 5 mM ATP	4.0
1 mM $MgCl_2$ + 10 mM ATP	1.0
2 mM $MgCl_2$	1.5
2 mM $MgCl_2$ + 1 mM ATP	14.0
5 mM $MgCl_2$	1.0
5 mM $MgCl_2$ + 1 mM ATP	12.0
10 mM $MgCl_2$	0.5
10 mM $MgCl_2$ + 1 mM ATP	10.0

Endonuclease activity was measured after gel filtration on Toyopearl HW 60F according to "Methods" with changes indicated.

Table 3. Summary of activities of endonucleases from rat liver nuclei

Enzyme	Yield of units from 100 g liver
Ca^{2+}, Mg^{2+}-dependent endonuclease	
Ca^{2+} + Mg^{2+}	10180
Mg^{2+}	2360
Mn^{2+}	5020
EDTA (pH 5.0)	4910
Ca^{2+} + Mg^{2+} + ATP	10300
Mn^{2+}-dependent endonuclease	
Ca^{2+} + Mg^{2+}	1160
Mg^{2+}	1320
Mn^{2+}	1950
EDTA (pH 5.0)	1000
Mn^{2+} + ATP	1880
Acid endonuclease	
Ca^{2+} + Mg^{2+}	830
Mg^{2+}	660
Mn^{2+}	530
EDTA (pH 5.0)	1310
EDTA (pH 5.0) + ATP	1300

Enzyme activities were measured after gel filtration through Toyopearl HW 60F as described in "Methods".

All calculations were based on data obtained with enzymes after gel filtration. To distinguish between the Ca^{2+}, Mg^{2+} - dependent and Mn^{2+}-dependent enzyme the synergistic effect of Ca^{2+} and Mg^{2+} ions was taken as a basis. For Ca^{2+}, Mg^{2+} dependent enzymes this ratio was higher then 2, while for the Mn^{2+}-dependent and acid nucleases it was lower than 2. ATP-stimulated endonuclease purified together with Ca^{2+}, Mg^{2+}-dependent enzymes but was separable from them. Endonucleases showed residual activities in all buffers used, however, optimal activity of each individual enzyme needed specific conditions. Based on the maximal activities detected under optimal conditions Ca^{2+}, Mg^{2+}-dependent, Mn^{2+}-dependent and acid endonucleases were shown to share 60, 20 and 10 % of the total activity, respectively.

DISCUSSION

A two-step chromatographic procedure was used for the separation of major endonucleases from rat liver and for the removal of other nucleases such as DNA topoisomerases and exonucleases. Three types of nuclear endonuclease were distinguished. Ca^{2+}, Mg^{2+}-dependent, Mn^{2+}-dependent and acid endonucleases were shown to share approximately 60, 20 and 10 % of the total nucleolytic activity, respectively. Minor nucleases represented the rest of the endonuclease activity.

Endonucleases investigated so far are known as Mg^{2+}-dependent ones. Our results show the characterization of an endonuclease is not satisfactory on the basis of its Mg^{2+} requirement only. The stimulation of nucleases by Mg^{2+} covers a broad range of more specific properties involving Ca^{2+}, Mg^{2+}, Mn^{2+}or ATP-dependency, topoisomerase activity, etc. Thus the possibility cannot be ruled out that nuclear Mg^{2+}-dependent endonuclease activity of mammalian cells (Yoshihara et al. 1974; Machray, Bonner, 1981; Tanigawa, Shimoyama, 1983) is at least partly due to Ca^{2+}, Mg^{2+}-endonucleases or to Mn^{2+}-dependent ones.

The properties of the Mn^{2+}-endonuclease described here are to some extent at variance with those reported by Yoshihara (1974) and Machray (1981) who have shown a considerable lower pH optimum of 5.5. In contrast to the Mn^{2+}-dependent enzyme from rat liver the endonuclease isolated by Tonigawa (1983) from hen liver nuclei has a higher molecular mass (M_r 43 $\pm$ 2 kD), a pH optimum of 9.0 and acts preferentially in the presence of denatured DNA. A Mn^{2+}-stimulated endonuclease cutting at neutral pH on denatured DNA substrate was described by Vinter (1983). In our hands both native and denatured DNA was nearly equally hydrolyzed by the Mn^{2+}-dependent enzyme (Bubnov et al. 1987).

To distinguish between Mn^{2+} and Ca^{2+}, Mg^{2+} endonucleases we suggest to use the term Ca^{2+}/Mg^{2+} synergism characteristic of the later enzyme only. The synergistic effect is a general property of the Ca^{2+}, Mg^{2+}-dependent endonucleases. It refers to the difference of nuclease activity which is higher in the presence of Ca^{2+} + Mg^{2+} than in the presence of Mg^{2+} alone.

Ca^{2+}, Mg^{2+}-dependent endonuclease (group V) was tested for its requirement of ATP. The demonstration of a minor ATP-requiring enzyme indicates that similarly to the ATP-dependent DNase in bacteria called recombination enzyme, such an entity may be present in higher organisms, too. This ATP-dependent Ca^{2+}, Mg^{2+} endonuclease is different from those of topoisomerase type II enzymes since it was separable from type II topoisomerases.

The presence of DNA-dependent ATPases in rat liver nuclei can be demonstrated only after intensive purification. These enzymes are localized in the chromatin of human lymphocytes as reported earlier Ohlbaum et al.(1979).

The standardization of measurements of basic levels of nuclear enzymes serves our future plans aiming at human diagnostic adaptation.

Acknowledgements

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AUTOPHOSPHORYLATION OF PHOSPHORYLASE KINASE AND ITS REGULATORY FUNCTION IN THE DEPHOSPHORYLATION OF PHOSPHORYLASE A

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SUMMARY

Autophosphorylation of phosphorylase kinase was measured under conditions that favoured autoactivation. Heparin and troponin C stimulated the autophosphorylation of phosphorylase kinase at pH 6.8 in a Ca^{2+}-dependent manner. The concentration required for the half-maximal stimulation of autophosphorylation for calcium ions was 2 μM in the absence of effectors, whereas 0.7 μM and 0.1 μM in the presence of troponin C and heparin, respectively. Calmodulin increased the rate of autophosphorylation of the α subunit only, resulting in a slight increase in the rate of autoactivation of phosphorylase kinase. Troponin C, heparin and polybrene enhanced the rate of autophosphorylation of both α and β subunits. The increased autophosphorylation coincided with an enhancement of kinase activity. Neither of these stimulatory macromolecules had significant influence on the total number of phosphate groups incorporated into the α or β subunits by autophosphorylation. Thio-autophosphorylated form of phosphorylase kinase behaved as an inhibitor in the dephosphorylation of phosphorylase a by the catalytic subunits of phosphatase-1 or phosphatase-2A and by the latent form of phosphatase-$2A_0$. Concentration of phosphorylase kinase needed to 50% inhibition was in the range of 0.05-0.08 μM.

INTRODUCTION

Phosphorylase kinase from rabbit skeletal muscle is known to be regulated by cAMP-dependent phosphorylation and calcium ion, therefore it has central importance in the hormonal and neural control of glycogenolysis (1). The enzyme is a hexadecamer of four different subunits with a composition of $\alpha_4 \beta_4 \gamma_4 \delta_4$ (2,3). The α and β subunits can be phosphorylated by either cAMP-dependent protein kinase or by phosphorylase kinase itself in a Ca^{2+}-dependent manner (4, 5). The phospho-

rylation of phosphorylase kinase significantly increases its enzymatic activity at physiological pH resulting in a concomitant enhancement in the affinity of phosphorylase kinase for protein substrates and Ca^{2+} (6, 7). It was also demonstrated that phosphorylase kinase, phosphorylated by cAMP-dependent protein kinase, caused an inhibition in the dephorphorylation of phosphorylase a by protein phosphatase, suggesting an additional function of this enzyme in maintaining of high level of phosphorylase a during glycogenolysis (8, 9). The α and β subunits are dephosphorylated by two types of phosphatase. Phosphatase-1 is rather specific for the dephosphorylation of the β subunit, whereas phosphatase-2A preferentially dephosphorylates the α subunit of phosphorylase kinase (10).

The α and β subunits have also been implicated in the interaction of phosphorylase kinase with its macromolecular effectors, such as extra calmodulin, troponin C (6, 11), glycogen and heparin (7). These macromolecules stimulate the activity of phosphorylase kinase in the phosphorylase b to a conversion (6), but they have diverse effects on the Ca^{2+}-dependency of the enzyme (12). Heparin and calmodulin can also modify the phosphorylation of phosphorylase kinase by cAMP-dependent protein kinase (13, 14). Previous studies have established that autophosphorylation of phosphorylase kinase is also influenced by macromolecular effectors and substrates of the enzyme. It was demonstrated that glycogen, glycogen synthase, polylysine, polymixins, phosphatidic acid and SDS markedly altered the rate of autophosphorylation and autoactivation of phosphorylase kinase. These effectors also increased the total number of phosphates incorporated into the α and β subunits (15-18,32). However, the physiological role of autophosphorylation of phosphorylase kinase has not been understood clearly.

We have examined the effect of exogenous calmodulin, troponin C, heparin and polycationic polybrene on the autophosphorylation of phosphorylase kinase with special interest in the Ca^{2+}-dependency of the autoactivation process. Furthermore, the inhibitory effect of autophosphorylated phosphor-

ylase kinase on phosphorylase phosphatase activities of phosphatase-1 and phosphatase-2A is also presented.

MATERIALS AND METHODS

Heparin (M_r 13,500-15,000) was purchased from Calbiochem. Heparin degradation product (M_r 4550) and heparinoid preparation (M_r 6500) were generous gifts from Organon. Calmodulin was obtained from Reanal (Hungary). Polybrene and ATP-γ-S were purchased from Serva, polyglutamate and polyaspartate from Sigma. (γ-^{32}P) ATP was obtained from Hungarian Isotope Institute. Heat-stable inhibitor protein of cAMP-dependent protein kinase was prepared from rabbit skeletal muscle according to the method of Walsh and Ashby (19). Rabbit skeletal muscle troponin C was kindly provided by Dr L. Muszbek (Department of Clinical Chemistry, University School of Medicine, Debrecen).

Phosphorylase kinase was purified as described by Cohen (5). The enzyme had a specific activity of 5.1 U.mg^{-1} at pH 8.2 and pH 6.8/8.2 activity ratio was 0.07. Phosphorylase b was obtained by the method of Fischer and Krebs (20) and was converted to phosphorylase a with phosphorylase kinase (21). Catalytic subunits of phosphatase-1 and phosphatase-2A were purified from rabbit skeletal muscle and separated as described previously (22). Partially purified latent phosphatase-2A$_o$ was prepared from rabbit liver (23).

Autophosphorylation of phosphorylase kinase was carried out at 30 °C in 40 mM Hepes/10 mM β-mercaptoethanol (pH 6.8). The reaction mixture (0.6 ml) contained 0.1 mg/ml nonactivated phosphorylase kinase, 10 μg/ml heat-stable inhibitor of cAMP-dependent protein kinase, 0.2 mM (γ-^{32}P)ATP, 10 mM Mg-acetate, 0.1 mM $CaCl_2$ and effectors where indicated. After 3 min preincubation, the reaction was initiated by addition of MgATP. The Ca^{2+}-concentrations of assay mixtures were adjusted by addition of $CaCl_2$ and EGTA as described previously (12). At intervals, aliquots of the reaction mixture were withdrawn, and analyzed for (^{32}P) phosphate incorporation or activity. The activity of phosphorylase kinase was assayed as described by Cohen (5).

(^{32}P) phosphate incorporation was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with 6 % gels according to Laemmli (24). Cylindrical gels containing the stained α and β subunits of phosphorylase kinase were excised and incubated with 30 % hydrogen peroxide (0.5 ml) in scintillation vials for 4h at 80-90 °C. After addition of 5 ml 0.1 M NaOH the radioactivity was determined by Cerenkov counting in a liquid scintillation spectrometer (8).

Thio-autophosphorylation of phosphorylase kinase was carried out in the presence of 0.2 mg/ml phosphorylase kinase and 1 mM ATP-γ-S. The autophosphorylation reaction (20 min) was terminated by addition 0.1 volume of 200 mM EDTA and the solution (1 ml) was dialysed against 10 mM Tris (pH 7.0) containing 0.1 mM EGTA and 0.1 % (v/v) β-mercaptoethanol. The activity of thiophosphorylated phosphorylase kinase was

5.7 $U.mg^{-1}$ at pH 8.2 with a pH 6.8/8.2 activity ratio of 0.35.

Phosphorylase phosphatase activity of the catalytic subunits of phosphatase-1 and phosphatase-2A was assayed with 0.4 mg/ml phosphorylase a at 30 °C in the presence of 40 mM Tris (pH 7.4). The reaction mixture also contained 0.1 mM EGTA, 10 mM β-mercaptoethanol, 5 mM caffeine and tio-autophosphorylated form of kinase in the concentrations indicated in figure legends. The reaction mixture also contained 50 µg/ml of polybrene when the activity of phosphatase-$2A_o$ was assayed. The phosphatase activity was determined by measuring the decrease in phosphorylase a activity caused by phosphatases as described previously (21).

RESULTS

Time course of the autophosphorylation and autoactivation of phosphorylase kinase is depicted in Fig. 1. The phosphate incorporation into the α and β subunits as well as the extent of autoactivation of phosphorylase kinase reached plateau values after 20 min in the absence of effectors. The presence of effectors modified only the initial rate of autophosphorylation, but they had no significant influence on the total amount of phosphates incorporated into the α and β subunits. Calmodulin increased the rate of phosphorylation of the α subunit without effecting that of the β subunit. The stimulatory effect of calmodulin was accompained with an increase (1.3-fold) in the rate of autoactivation of phosphorylase kinase. Increasing the concentration of calmodulin 10-fold (35 µg/ml) in the autophosphorylation reaction did not cause further stimulation of phosphorylation of either subunits, in fact a slight inhibition of phosphorylation in the β subunit could be observed (data not shown). Troponin C and heparin enhanced the rate of phosphorylation of both the α and β subunits as well as the rate of autoactivation by more than twofold.

The experiments described in Fig. 2 show the effect of calmodulin, troponin C and heparin on the Ca^{2+}-dependency of the autophosphorylation of phosphorylase kinase as measured by the incorporation of (^{32}P) phosphate into the α and β subunits of kinase. Calmodulin slightly stimulated the initial rate of autophosphorylation in the presence of micromolar

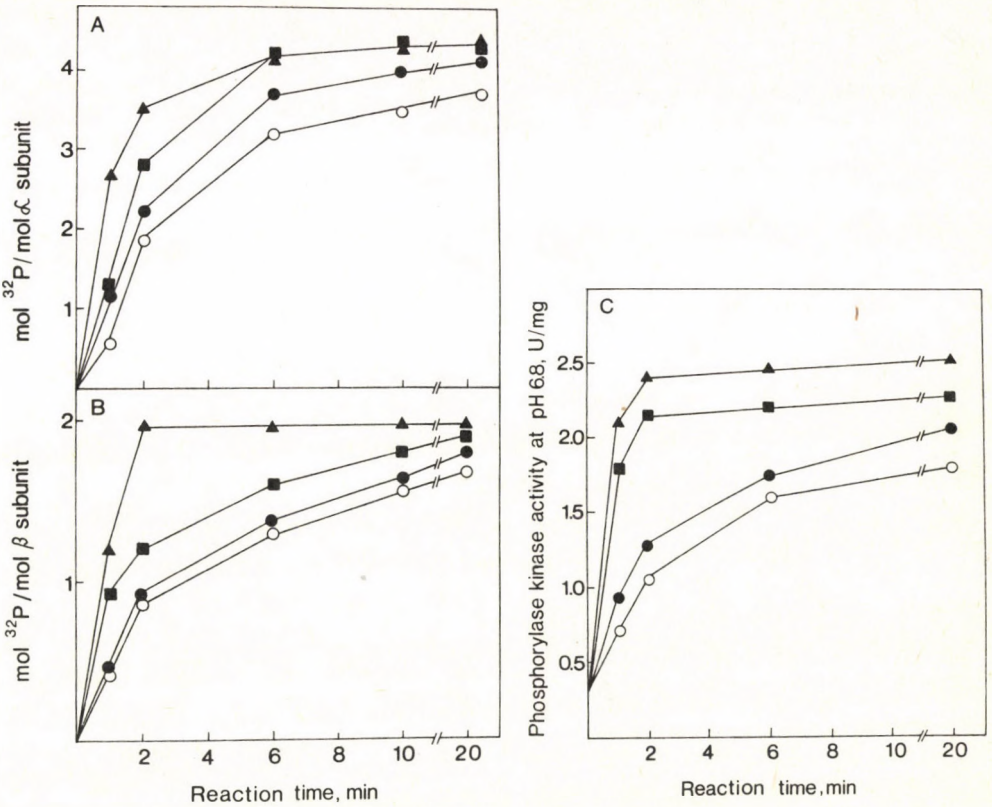

Fig. 1. Time course of the autophosphorylation and autoactivation of phosphorylase kinase. The incorporation of (^{32}P) phosphate into α (A) and β subunit (B) of phosphorylase kinase and the effect of autophosphorylation on the activity of the enzyme at pH 6.8 (C) were determined as given in Methods. Additions to reaction mixture were: none (o), 3.5 μg/ml calmodulin (●), 100 μg/ml troponin C (■) and 50 μg/ml heparin (▲).

concentration of free Ca^{2+}, with a half-maximal effect occurring at 2 μM of Ca^{2+}. Troponin C enhanced the rate of autophosphorylation in a Ca^{2+}-concentration range (0.1 μM - 100 μM), half-maximal stimulation was developed at 0.7 μM of Ca^{2+}. Heparin markedly stimulated the autophosphorylation when the concentration of free Ca^{2+} was lower than 0.1 μM,

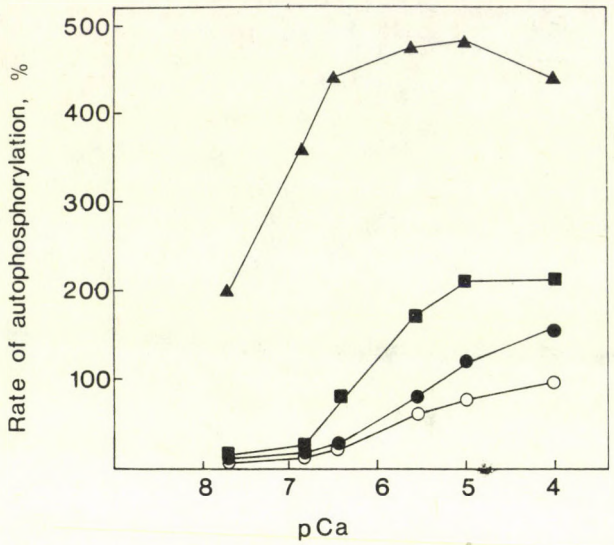

Fig. 2. Ca^{2+}-dependency of autophosphorylation of phosphorylase kinase at pH 6.8. The initial rate of autophosphorylation of phosphorylase kinase was determined as described in Methods in the presence of various concentrations of Ca^{2+}. Initial rate in the absence of effectors and presence of 100 μM Ca^{2+} was taken as 100 %. Additions to the assay mixture were: none (o), 3.5 μg/ml calmodulin (●), 100 μg/ml troponin C (■) and 50 μg/ml heparin (▲).

and its stimulatory effect was more expressed by increasing the concentration of Ca^{2+}. In the presence of heparin half-maximal activation of autophosphorylation by Ca^{2+} could be attained at 0.1 μM of free Ca^{2+}. It may be noted that there was no significant difference in the Ca^{2+}-dependency of the autophosphorylation of the α or β subunits.

It has been shown that heparin stimulation of liver phosphorylase kinase is rather specific for this polysaccharide and its effect are not mimicked by closely related polysaccharides (25). Heparin preparations of different molecular weights, heparinoid compound and charged polymeric molecules

Table 1. Effect of polyanionic and polycationic macromolecules on autophosphorylation of phosphorylase kinase

Activation medium contained 100 μM Ca^{2+} and effectors in 50 μg/ml concentrations. The initial rates of phosphorylation of the α subunit (0.6 mol $^{32}P \cdot min^{-1}$) and the β subunit (0.39 mol $^{32}P \cdot min^{-1}$) in the absence of effectors were taken as 100 %. All values represent three independent experiments (SD ± 10 %). Calbiochem and Organon 31121 preparations represent pure heparin with different molecular weights. Organon 10172 is a heparinoid of natural origin containing about 5 % of low molecular weight heparin-like component, 80 % heparan sulfate, 11 % dermatan sulfate and 4 % chondroitin sulfate.

Additions	Rate of phosphorylation (%)		Autoactivation (%)
	α subunit	β subunit	
none	100	100	100
Heparin (Calbiochem), M_r 13,500-15,000	468	241	450
Heparin (Organon 31121), M_r 4550	306	164	235
Heparin (Organon 10172), M_r 6500	231	125	182
Polyaspartic acid, M_r 20,000	86	97	92
Polyglutamic acid, M_r 14,000	106	100	100
Polybrene	396	252	425

were introduced in the study of autophosphorylation of phosphorylase kinase to gain further information about the mechanism and specificity of the action of heparin. These data are shown in Table 1. It is clear that the stimulatory effect of heparin on the autophosphorylation and autoactivation is reduced by decreasing its molecular weight. In the presence of heparin-like compound the stimulatory effect is further reduced. Polyglutamate and polyaspartate, similar to heparin in their polyanionic character and molecular weight, were without effect on the autophosphorylation and autoactivation. It is of interest that polybrene, a positively charged artificial macromolecule, profoundly increased the rate of autophosphorylation and autoactivation reactions.

It was previously demonstrated that phosphorylase kinase, thiophosphorylated by cAMP-dependent protein kinase in the presence of ATP-γ-S, exerted an inhibitory effect on the phosphorylase phosphatase activity of protein phosphatase (8). The effect of autophosphorylated phosphorylase kinase on the phosphorylase phosphatase activities of different types of phosphatase has not been investigated so far.

Fig. 3 demonstrates the concentration-dependent inhibition of phosphorylase phosphatase activities of the catalytic subunits of phosphatase-1 and 2A by the thio-autophosphorylated-form of phosphorylase kinase. Phosphorylase kinase also exerted an inhibition on the activity of latent (high molecular weight) form of phosphatase-$2A_o$. It is seen that both types of phosphatase were inhibited by thio-autophosphorylated phosphorylase kinase and 50 % inhibition of phosphatase-1 and phosphatase-2A activities could be attained in the presence of 0.05-0.08 μM of phosphorylase kinase.

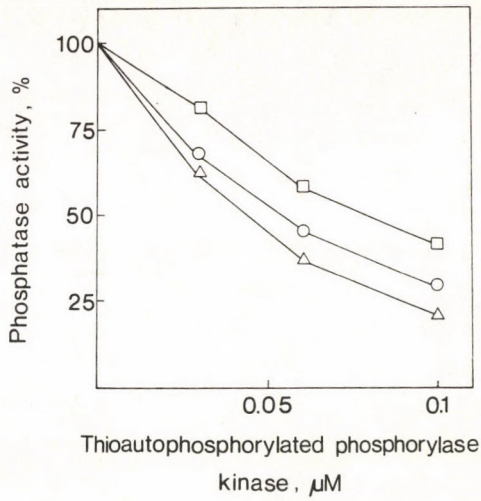

Fig. 3. Inhibition of phosphorylase phosphatase activity of protein phosphatases by thio-autophosphorylated phosphorylase kinase. The activity of phosphatase -1 (o), phosphatase-2A (Δ) and polybrene-activated latent phosphatase-$2A_o$ (□) was determined as described in Methods in the presence of different concentrations of thio-autophosphorylated form of phosphorylase kinase. Phosphatase activity was taken as 100 % in the absence of phosphorylase kinase.

DISCUSSION

Our present results demonstrate that calmodulin, troponin C, heparin and polycationic polybrene exert diverse effects on the autophosphorylation of phosphorylase kinase in respect to their abilities to influence Ca^{2+}-dependency of the process and to increase the initial rate of phosphorylation of the α and β subunits (see Fig. 1 and 2). Previous studies showed that calmodulin, troponin C and heparin also stimulated the conversion of phosphorylase b into a catalysed by phosphorylase kinase (6, 12, 26, 27). The effects of these macromolecules on the autophosphorylation clearly indicate that they can bind to phosphorylase kinase, and the diversity of their action supports the view that their interaction with the enzyme is different.

Calmodulin and troponin C show 50 % identity in their amino acid sequences and their Ca^{2+}-binding abilities are also similar (28). They stimulated the conversion of phosphorylase b into a to nearly identical extent at saturation Ca^{2+}-concentrations, but their effect on the Ca^{2+}-dependency was different (6, 12). Similar difference was found in the autophosphorylation reaction (see Fig. 1), since troponin C decreased the Ca^{2+}-concentration required for half-maximal activation of phosphorylase kinase, whereas calmodulin was without effect on the Ca^{2+}-dependency. This latter finding is in agreement with the earlier proposal of Burger et al. (29), namely that intrinsic Ca^{2+}-binding properties of phosphorylase kinase do not change upon binding exogenous calmodulin. The differences in the interaction of phosphorylase kinase with calmodulin and troponin C are also revealed by studying the phosphorylation of the α and β subunits and autoactivation of the enzyme in the presence of these effectors. In accordance with earlier results (14, 27, 30) calmodulin slightly increases the rate of autophosphorylation and autoactivation of phosphorylase kinase. Our present data indicate that this stimulation is due to an increase in the α subunit phosphorylation whereas the β subunit phosphorylation remains unchanged in the presence of calmodulin. This observation is consistent with the assumption that binding of calmodulin to phosphorylase kinase may alter differently the accessebility of Ser residues of the α and β subunits to autophosphorylation. Evidence for a similar alteration of phosphorylation of the α and β subunits by cAMP-dependent protein kinase in the presence of Ca^{2+} and calmodulin was obtained by Cox and Edstrom (14). Troponin C enhanced the rate of phosphorylation of both the α and β subunits, accompanied with a more pronounced increase in the rate of autoactivation. The above results favour the assumption that phosphorylase kinase activation by autophosphorylation is mainly correlated with the phosphorylation of the β subunit of the enzyme (1, 5).

The stimulation of autophosphorylation and autoactivation by heparin appears to be specific for this polysaccharide

since a heparin-like compound (Organon 10172), containing closely related sulfated polysaccharides, is less effective and anionic polyamino acids cannot mimic the effect of heparin (see Table I). In contrast to calmodulin and troponin C, heparin stimulated the autophosphorylation in the presence of lower concentrations of Ca^{2+} suggesting a Ca^{2+}-independent interaction of heparin with phosphorylase kinase. This view is also supported by the data of Meggio et al. (13) that heparin has a substrate-directed effect in the phosphorylation of phosphorylase kinase by cAMP-dependent protein kinase in the presence of EGTA. Phosphorylase kinase activity assayed by the conversion of phosphorylase b into a was not stimulated by 50 μg/ml of heparin at Ca^{2+}-concentration lower than 0.1 μM (12). On the other hand, heparin, over 1 mg/ml, markedly increased the Ca^{2+}-independent activity of phosphorylase kinase but it was without effect on the autophosphorylation reaction (31). This high concentration of heparin caused the dissociation of phosphorylase kinase (31), whereas heparin, below 50 μg/ml, increased the activity of holoenzyme at pH 6.8 (7) in a Ca^{2+}-dependent manner (13). This latter finding also excludes the dissociation of phosphorylase kinase by heparin of lower concentrations. It may be concluded that heparin stimulation in the autophosphorylation and autoactivation reactions of phosphorylase kinase depends upon the protein substrate, the concentrations of heparin and Ca^{2+}, too.

The autophosphorylation of phosphorylase kinase was shown to be influenced by polycationic macromolecules, such as histones (30) and polylysine (16), moreover by polymixins (17) and phosphatidic acid (18). In this study polybrene, a heparin antagonist polycation, proved to be an effective activator both in the autophosphorylation and in the autoactivation of kinase. Polybrene increased the initial rate of autophosphorylation of both subunits without affecting the total number of phosphate incorporated into phosphorylase kinase. In this respect its stimulatory effect markedly differs from polylysine, since polylysine was found to increase the total number of phosphate groups incorporated into the α and β

subunits by autophosphorylation, and this elevation of phosphate content resulted in the inactivation of the enzyme (16). The stimulation of autophosphorylation by polymixin B was accompanied with an enhancement of the rate of autoactivation at pH 6.8 (17). Furthermore, polylysine and polymixin B were potent inhibitors of phosphorylase kinase (16, 17), whereas polybrene had no effect on this reaction (unpublished observation of Erdődi et al.).

Two types of phosphatase, namely phosphatase-1 and phosphatase-2A, possess considerably activity towards both phosphorylase kinase and phosphorylase a. Our present data indicate that the autophosphorylated form of phosphorylase kinase can inhibit phosphorylase phosphatase activity of both types of phosphatase (Fig. 3) and 0.05-0.08 μM of kinase is needed to the 50 % inhibition. It has been demonstrated that phosphorylase kinase phosphorylated by cAMP-dependent protein kinase inhibited the dephosphorylation of phosphorylase a by protein phosphatases with an apparent K_i of 0.1 μM (8, 9). It seems that the rise in the phosphate content of kinase by autophosphorylation only slightly enhances its inhibitory capacity. Phosphatase-1 and 2A dephosphorylate phosphorylase kinase with different specificity. Phosphatase-2A seems to be specific for the dephosphorylation of the α subunit (10). The exact function of the phosphorylation-dephosphorylation of the α subunit has not been clear. According to our present result the phosphorylation of the α subunit may be important in the regulation of phosphorylase phosphatase activity of phosphatase-2A.

The occurrence and physiological significance of autophosphorylation of phosphorylase kinase in vivo are debated. It was postulated that activation of nonactivated form of phosphorylase kinase by Ca^{2+} is responsible for the coupling between contraction and glycogenolysis (6). In this context, Cohen (6) reported that troponin C or troponin complex may be a Ca^{2+}-dependent phisyological activator of phosphorylase kinase. It is an enigma, whether the stimulation of phosphorylase kinase autoactivation by heparin or polycationic

polybrene may mimic a regulatory device actually occurring in vivo. Troponin C is one of the candidates which may represent a real stimulatory molecule in the autoactivation of phosphorylase kinase in vivo. In addition, autophosphorylation os phosphorylase kinase might be involved in the regulation of phosphorylase phosphatase activity of different types of phosphatase, contributing to the maintenance of phosphorylase a level during glycogenolysis.

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THE ORIGIN OF BIOPOTENTIALS ACCORDING TO THE PHASE BOUNDARY CONCEPT

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The membrane theory

Until the beginning of our century the biopotentials were explained by theories in which the proteins sometimes played a prominent part (Du Bois-Reymond, 1848-1884). In contrast to them, Bernstein (1902) used in his membrane theory only elements whose properties were sufficiently well known to allow a mathematical treatment, namely the water and the strongly dissociated small ions. He did not deny the possible influence of intracellular proteins, which interact strongly with water as well as with small ions, with the intracellular structures, which might influence the ionic distribution, or with the electric component of the biochemical activity in the resting cell. But at that time all these functional elements were presumed rather than experimentally observed or measured. Excluding them from his consideration Bernstein put forward a theory of the origin of bioelectric potentials, based upon a solid physicochemical knowledge, permitting a quantitative interpretation of the experimental data.

This exclusion of important electrogenic sources ineluctably leads to contradictions with the experimental data. Accordingly, the membrane theory underwent serious modifications. It had to be admitted that even the resting membrane has a fair permeability to cations, including sodium ions, and thus requires metabolically driven ion pumps to maintain the ionic disequilibrium between the cell and the outer medium. But the electrogenic system is still regarded to be composed of two weak solutions of cations moving freely in completely free water and separated by a semipermeable membrane. Still no influence is assigned to the intracellular proteins. Therefore, there still remain profound contradictions between the membrane theory and the experimental facts. They have been exhaus-

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tively analysed e.g. by Ling (1962) and Segal (1978, 1983).

The alternative way would be to take into consideration the intracellular proteins. Their electrical properties and their interaction with ions and water molecules have meanwhile become well known. In this respect, the cytoplasm appears as a phase qualitatively distinct from the outer medium, in which ions are accumulating according to different laws than those that prevail in watery solutions. The boundary between these two phases must display electrogenic properties.

There are different ways of dealing with this problem. Ling (1984) formulates an "association induction hypothesis" taking into account the strength of interaction between proteins, ions and water molecules; Kurella (1969) considers the proteins to be ion exchangers; Segal (1978, 1983) describes the cytoplasm as a coacervato with strongly bound intermolecular water bridges and an affinity to potassium ions in preference to sodium ions.

It remains to be decided which of these approaches is correct. For the time being we are awaiting with interest the consequences which may result from any of them. The final solution might be a synthesis from elements of different phase boundary theories.

In the present paper we shall explain the consequences which would result if we agreed to consider the cytoplasm as a protein coacervate.

The structure of globular protein molecules

When investigated by the modern method of X-ray analysis with multiple substitution of heavy atoms, most globular protein molecules appear as hollow drumlike structures, open at both ends (Richardson, 1977). As early as 1960 Segal et al. (1960) postulated that this form, which they called the Faltentrommel model, was the basic structure which, in multiple topological transformations, is adequate to differentiate functional properties of spheroproteins. In a modernized form, the Faltentrommel model is described by Segal et al. (1983 annex 2). Up to now this model is the only one to provide a relation between the physico-chemical or physiological properties of globular proteins and their structure. We cannot but choose it as the basis of our attempt to understand the relation between proteins, ions and water inside the cell.

The wall of the drum is usually described as a cylindrically bent β-pleated sheet with linear peptide strands alternatively antiparallel,

thus totally cancelling the dipole moment of the structure. In the Faltentrommel the wall is formed by parallel strands, the 180° bend between them being realized by diketopiperazines. The peptide bonds are all directed in the same way, and the sum of their dipole moments provides a global dipole moment, parallel to the molecular axis (Fig. 1).

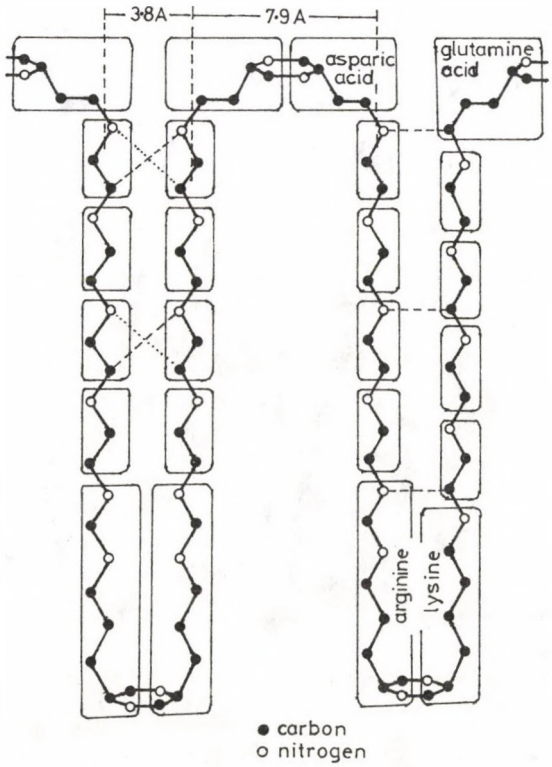

Fig. 1 The wall of a "Faltentrommel molecule showing the possibilities of hydrogen bonds within the narrow folds. The conditions in the right narrow fold are more frequently realized.

The amino acid residues forming the peptide strands have the L-configuration and all bear an asymmetrical electric charge, resulting in a slight spiral twist of the peptide chain by about 3° per residue (Law, 1953). The molecular wall grows therefore with a cylindrical twist, and when the total twist reaches 360°, the cylinder is closed and can grow no

more (Fig 2). That is why the normal monomeric molecular weight is usually near 15,000 D, corresponding to some 120 residues. Somewhat higher monomeric weights arise when prosthetic groups interfere with the regular growth of the drum and add their molecular weights. Thus, the monomeric myoglobin molecule rises to 17,000 D. A further increase of the molecular weight results from the association of several monomers: 2 in actin, 4 in hemoglobin, 12 in erythrocruorin.

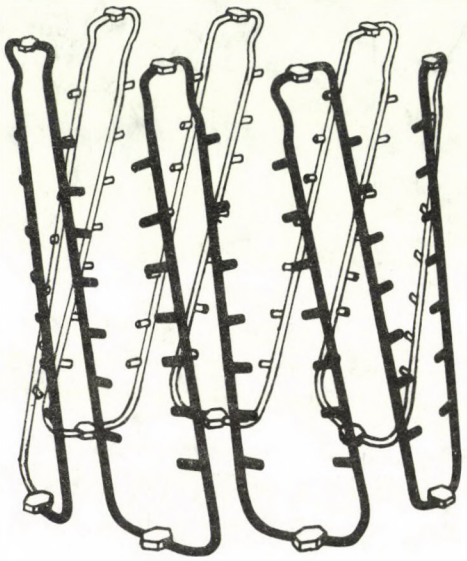

Fig. 2 A molecule of the purple membrane of Halobacterium halobium, interpreted as a Faltentrommel. It bears 14 linear peptide chains, slightly spiralized and inclined by about 30^{o} with respect to the molecule axis. At the inflexion points the hexagonal diketopiperazine rings are visible

Topological transformations of this basic structure can account for multiple functional adaptations of a protein. The section of the drum may be cylindrical, providing an axial pore 20 Å wide, such as we observe in the actin-like membrane penetrating proteins. In the ribonuclease the lumen of the molecule is constricted by transversal S-S-bridges, in order to adapt it to the configuration of the substrate. In the immunoglobulins the lumen is completely flattened, thus providing a rigid and stable binding site. In this class of proteins, the terminal monomer is highly

distorted in order to adapt the structure of the binding site to the configuration of the antigen, but topologically it still remains a Faltentrommel (Capra, Edmundson, 1977).

Another topological transformation results from the varying length and number of the linear peptide chains. In the myoglobin molecules the required number of 3^{0} twists is realized by 28 linear chains of 5 residues each, forming a very short cylinder with a wide lumen giving easy access to O_2 molecules. The molecules of the membrane proteins consist of 2 monomers in a head-to-tail conformation. Each chain has the length of 10 residues, of about 36,5 Å, and two such molecules form a tube of 73 Å, just enough to penetrate completely a 70 Å phospholipid double layer. On the other hand, the 360^{0} bend requires only 12 peptides of these long chains, and the channel so formed has a lumen of only 20 Å. Calcium ions bound to the inner surface of this channel may extend their electrostatic field across the whole width of the lumen and thus regulate the cation permeability of the membrane.

The Faltentrommel bears most of its hydrophilic groups at the two rims of the monomer. In the myoglobin, the linear chains are short, and the molecule is strongly hydrophil. In the membrane proteins the linear chains bearing hydrophobic groups are long. They form, between the two rims, a hydrophobic belt, which is likely to interact with the hydrophobic chains of the phospholipids. Accordingly, the width of the hydrophobic belt is 28,8 Å, while the hydrophobic tails of the most frequently encountered membrane phospholipids are 28 Å long.

Due to its high dipole moment, the dimeric molecule is oriented vertically to the cell surface, the hydrophobic belt is attracted by the lipidic tails of the phospholipid molecules, and the protein molecule forms a hydrophilic channel penetrating the lipid membrane (Fig. 3).

The serum prealbumin is the protein which carries hydrophobic hormones along the watery blood stream. Its molecule is formed by 4 identical hydrophilic drum-like monomers, but each of these carries a short α-helix antenna, a highly hydrophobic structure able to capture lipidic molecules.

Such relations between the structure of a protein molecule and its functional properties will guide us in the discussion of the interaction between globular proteins, ions and water within the cytoplasm and in the plasma membrane.

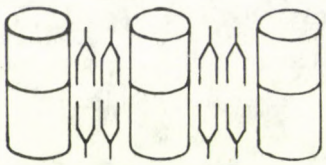

Fig. 3 The disposition of the actin-like protein and the phospholipid molecules inside the plasma membrane of a cell

Proteins and water in the hyaloplasma

Most of the proteins forming the hyaloplasma are globulins and other globular proteins with an antiparallel structure. They have been described as neuroglobulins. They are tetrameric molecules formed by a pair of dimers in head-to-tail position, each one with a high dipole moment, but put together in antiparallel orientation, so that most of the dipole moments are neutralized. They are only slightly acidic, with an isoelectric point around pH 6.7 or 6.8 (Depending on the ionic strength). At a slightly alkaline pH, like that prevailing in a resting cell, and in the presence of a suitable amount of cations, such proteins have a tendency to form coacervates.

We can describe a coacervate as the macromolecular equivalent of a fluid crystal of the nematic type (Cistjakov, 1966). A model of the coacervate structure is shown in Fig. 4. In this model the protein molecules cling together by their global negative field overlapping the positive pole of the dipole field, without touching each other, thus forming micella of oriented but easily dissociating protein molecules, which explains the fluidity of the structure. The overall negative charge of the micella is compensated by an equivalent amount of cations, in biological systems mostly potassium ions, linked to the protein by short chains of one, two or three water molecules.

These molecules are bipolarly bound by dipole fields. Both their poles being occupied, they have no solvating power. Furthermore they cannot move under thermic forces, but are limited to oscillations. They behave neither as fluid water, nor as hydration water, but as ice, the best approximation being given by the ice VIII, or crystal water inside a fluid crystal. At any rate such water must display a strongly reduced T_1 and a very slow diffusion.

This part of the tissue water forms compartment A: coacervate water

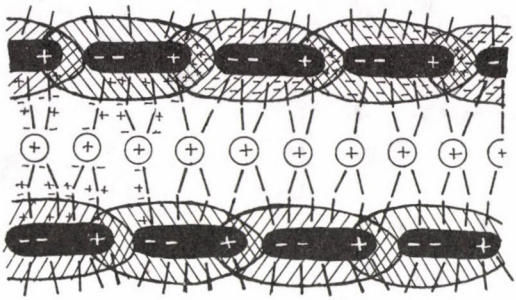

Fig. 4 Segal's model of coacervate structure. It shows the distribution of the electrical fields in the protein molecules, the global negative fields which surround them, and the small cations attached to them by bipolarly bound water molecules

in bipolar binding. Estimates, rather than exact measurements, make us believe that it amounts to some 70 to 80 per cent of the water in most tissues. It depends greatly upon the nature of the tissue and also upon its functional state.

Besides the globulins, each cell contains a fair amount of actin-like proteins. They are head-to-tail dipoles with a strong dipole moment (Segal, Kalaidjiew, 1977). This enables them to form transitory structures such as microfilaments, microtubules, cytoskeletons and mitotic spindles. Furthermore, they are strongly acidic. Thus, neither by their global charge nor by their dipole fields do they fit into a globulin coacervate. When they do not cling together by cooperative association, they seem to swim in the fluid coacervate in monomolecular dispersion.

In this state they behave as proteins dissolved in water, with the usual hydration layers. This water forms another compartment, compartment B. This water has the usual properties of water molecules bound to proteins in a monopolar mode, and the T_1 must be somewhat higher.

There are other proteins which contribute to compartment B. There is a certain amount of freely dissolved enzymes; there are proteins synthetized to be secreted, like antibodies in the lymphocytes or lactoproteins in the mammary gland.

Naturally, the value of compartment B changes with the physiological

conditions as well. Glands secrete only when they are required to, and actins lose much of their water binding power when their strongly hydrophilic rims are masked by combining into multimolecular chains. We cannot say how large compartment B is, but we would not be surprised if the measured values were somewhat less than 20 per cent.

But there is also a compartment C; the water with dissolved micromolecules and ions, but devoid of proteins. Such a fluid fills the intercellular clefts. They are usually 200 Å wide, and if we assume a tissue formed by 10 µm cubic cells, they may reach some 0.6 per cent of the whole volume of the tissue.

A second part of compartment C is composed of the intracellular channels such as the Golgi apparatus, the cytoplasmatic reticulum and the very ample sarcoplasmatic reticulum system in the striated muscle. Considering furthermore that in the muscle for instance much space is occupied by solid state structures, actin and myosin fibers and transversal membranes, we may expect that in some tissues compartment C may reach a rather high percentage. Together with the intercellular clefts compartment C may account for 1 per cent or even more.

It is not impossible that irregularities in the coacervate structure leave some amount of non-coacervated space, which may also add to compartment C. Working with artificial coacervates, Troschin (1966) found that a few percent of a dissolved substance enter quite quickly into the coacervate droplet, while the rest requires much more time. This may indicate the possibility that such a non-coacervated space exists. Then we ought to consider it also as part of compartment C. At any rate, the total amount is not supposed to surpass 4 or 5 per cent of the global tissue water.

Logically, the proportion of the three compartments must depend to some extent on the nature of the tissue investigated and on its functional state. A great part of the volume of a striated muscle fiber is occupied by the sarcoplasmic reticulum. Thus, compartment C ought to be larger than in other tissues. Furthermore, in the muscle the actin is permanently present in the form of filaments with masked hydrophilic zones. Therefore it binds little hydration water, and compartment B must be somewhat reduced.

Fortunately, we possess numerous measurements of the states of water in different tissues (Reisin, Ling, 1973; Belágyi, 1975; Masszi et al.,

1976). All the methods - self-diffusion, vapour pressure, paramagnetic resonance and microwave relaxation - show, that only a small amount of the tissue water is free, a somewhat greater amount is bound in a way corresponding to hydration sheets, while 70 per cent or even more is strongly immobilized, in a way hitherto non described by physicochemistry. It might well correspond to the bipolar binding of water molecules in a coacervate structure.

In agreement with our views Hazlewood (1977) found in the skeletal muscle the very high amount of 7.6 per cent of free water (compartment C), an unusually small compartment B of only 7.2 per cent and the usual compartment A of 85 per cent.

The same author studied various tumors. In this kind of tissue a fair amount of the cells are paranecrotic, with coacervates broken down and with a heavy loss of the intracellular potassium. Accordingly compartment A is reduced and compartment B increased.

Recently the school of Cameron (Merta et al., 1986; Fullerton et al., 1986) using the NMR method, found a fraction of "superbound" water, amounting to about 5 per cent of the global cellular water, which is independent of the physiological state of the cell. Since 1960 (Segal et al., 1960) we have emphasized that most of spheroproteins form hollow drum-like structures surrounding a water-filled lumen. This water is strongly bound to the protein molecule, as demonstrated by the unequal shrinking of the three crystal axes of spheroprotein crystals. Depending on the type of the protein the intramolecular lumen may occupy more or less than half of the molecular volume, and in cells with 10 to 15 per cent protein in the cytoplasm the part of the superbound intramolecular water is likely to form some 5 per cent of the water content of the cells.

This superbound water is found in living cells (Merta et al., 1986) as well as in protein solutions (Fullerton et al., 1986). It is part of the molecular structure and has apparently no relation to supramolecular systems such as coacervates or vesicles. It is so strongly bound that it does not interact with the other water compartments during the excitation cycle. One might describe it as a fourth compartment, but we prefer to reserve the term "compartment" only for the compartments A, B, and C, described above, which form an entity with strong dynamic interchanges.

The plasma membrane (Segal, 1978; Segal et al., 1983; Segal, Kalaidjiew, 1977).

Before discussing the status of electrolytes inside and outside the cell, we must pay some attention to the membrane which separates the two media. The theory of Bernstein required a semipermeable membrane, letting the K^+-ions pass and retaining the Na^+. It was concluded that the membranes have pores of 4 Å, but there was never any experimental evidence for this. When the ion migration was studied with radioactive isotopes, it was quickly shown that Na-ions penetrated the membrane. The Cambridge team still supported the semipermeability theory on the basis, that the K^+ influx had the value of $5{,}4 \times 10^{-12} mol/cm^2/sec$ while Na^+ influx had only 3.5 units (in the frog muscle). But if we consider that the equivalent conductance of the K^+ at 18^oC is 64.6 and that of Na^+ is only 43, we must conclude that the difference observed is due to the different degrees of mobility of the two ions in free water, and that the membrane does not exert any selective action upon them.

We know that the membrane is composed of hollow cylindrical molecules of the actin type, separated by phospholipid molecules in a bilayer disposition in the plasma membrane, most frequently in the arrangement shown in Fig. 3. This model is consistent with the functional data as well as with the electronmicroscopic evidence (Cartant et al., 1978).

Most modern authors agree that the membrane pores are 20 Å wide, corresponding to the inside space of an actin dimer. Glucose, ethidiumbromide, hemoglobin monomers, RNA and even DNA pass these pores. In spite of these facts the Cambridge school introduced specific K and Na pores, without explaining why these substances do not simply pass through the wide - open 20 Å pores and prefer the pores with a specific gating.

The answer to the question why the small cations are so reluctant to pass the membrane pores of 20 Å was given by Tasaki (1978) who calculated, that the presence of 2 Ca^{++} ions in each pore would be sufficient to explain such a low permeability for cations, while leaving free passage to glucose, water and other neutral molecules of higher diameter. We have identified the two binding sites for Ca^{++} inside the membrane pore. They are $C = O^{(-)}$ groups near the positive pole of each monomer.

When, during excitation, the polarity of the membrane is reversed, the peptide bond to which this carbonyl group belongs passes into the enolic form $C\text{-}O\text{-}H^{(+)}$ which no longer retains the positive calcium ion. The

resistance of the membrane breaks down to 1/50 of its initial value.

Several authors (Schindler, Rosenbusch, 1978; Tverdislow et al., 1980; Fink et al., 1980) studied the action of Ca^{++} on membrane resistance, in vivo and in artificial protein-lipid membranes. They all state that the supression of calcium results in an increase of permeability by a factor of 50. They all emphasize also that these changes correspond unspecifically to the K^+ as well as to the Na^+ permeability. Thus both ions migrate through the same kind of pores, and there is absolutely no need to invent separate sodium and potassium channels. The problem of the assumed two separate gating systems will be discussed farther on, in relation to the excitation mechanism.

As to the low permeability of the membranes to anions Bethe (1952) explained this as early as in 1952 by the acidic nature of the membrane proteins. Recent investigations confirmed that they do indeed have about twice as many acidic aminoacid residues as basic ones; thus their pores must be highly resistant with regard to anions (Bryan, 1972).

The distribution of small ions in the resting cell

By equilibrium dialysis of ion-plus-protein solutions against ion-without-protein solution we found three exchange compartments. Compartment I corresponds to freely dissolved cations. In our apparatus their equilibrium was reached within 6 minutes. Compartment II is formed by cations adsorbed on proteins. The binding points are mostly the carbonyl groups with a negative dipole field protruding outside the molecule. The exchange of these ions takes some 6 hours (Kunze, 1966).

Compartment III is formed by cations bound inside the molecule of protein probably bridging a weak hydrogen bond. With previously electro-dialysed proteins, the ions of this compartment take hours to occupy their sites and do not display any significant interchange even after days of dialysis.

The measurements were made at 1 per cent protein concentration. Extrapolation to 15 per cent corresponding to an average cell plasma will show for compartment I (free ions) 1-2 per cent for compartment II (adsorbed ions) nearly 50 per cent and for compartment III (incorporated ions) nearly 50 per cent.

Similar measurements in vivo, performed in frog muscle in Cambridge (Harris, 1953) yielded a fraction about 2 per cent K^+ dialysing to

equilibrium within 10 minutes, a second fraction with a time constant of several hours corresponding to about half of the cell potassium, and a third fraction, also about 50 per cent, which is released only after the death of the tissue, probably when autolysis sets in.

In the protein solution we detected only slight differences between the different univalent cations. Things changed with coacervation. In order to be introduced into the coacervate pattern a cation must be previously dehydrated, and this occurs with a potassium ion at much less energy expense than with a sodium ion. In fact, we found that a coacervate formed in a sodium-potassium medium incorporated the potassium selectively. Coacervates formed in sodium exchanged it quickly for potassium, while potassium coacervates did not exchange it for sodium (Martin, 1967).

Thus the potassium of compartment II accumulates in the coacervate and maintains its concentration, once the equilibrium level is reached, without requiring any semipermeable membrane or selectively working ion pump.

As to the small amount of intracellular sodium, it may result from an equilibrium of the unequal affinities of coacervates to sodium and potassium ions. But most likely, a part of it is simply dissolved in what we have called water compartment C, which corresponds mainly to the protein-free liquid in the cytoplasmic reticulum, in which it may have the same concentration as in the outside medium, but which represents only a small fraction of the global volume of the cell.

The anion amount inside the cell must be small. There are few anion-binding sites, due to the inaccessibility of the positive pole of the carbonyl dipole $^{(+)}C{=}O^{(-)}$. Furthermore, the positive dipole field of $^{(-)}N{-}H^{(+)}$ is much weaker than that of the carbonyl group, due to the smaller distance between the atom centers, and their binding energy to anions does not seem to overcome the energy of thermic agitation.

We now understand the mystery of the "missing anions" inside the cell. Protein-bound and coacervate-bound cations have nearly no ionic activity. They need no free anions to balance it, and there are no binding sites for anions on the protein. There is only slight cationic activity in water compartment III, and the slight chlorine concentration in the cell is certainly sufficient to compensate it. The cell is in equilibrium with the outer medium with regard to anions as well as with regard to cations.

In the resting state there is no net ionic flux in either direction and no diffusion potential.

The resting potential

Under the given conditions, with no net fluxes between the media and with an indifferent membrane, we cannot expect any diffusion potentials to arise. The only electrogenic element would be the contact between an outside phase with high ionic activity and an inside phase with low ionic activity. Here we must expect a potential gradient, a potential detected only by electrometric methods, since it is not accompanied by a current flow. Under physiological ionic concentrations it ought to reach some 30 mV, and in fact it is detected in coacervates as well as in living cells, squid neuron or fungal mycelia, whenever the cell respiration is blocked. In contradiction to Hodgkin, we must emphasize that even in his own measurements, the potential of a cell deprived of ATP does not fall to zero but only to 30 mV.

In more remote times resting potentials were measured by galvanometers, devices requiring a certain amount of current, and that means that, besides electrometric potentials, there are real ionic fluxes, more precisely cation effluxes.

An explanation of this apparent contradiction is given by the group of Slayman (Slayman,Gradmann, 1975; Slayman et al., 1973). In fungal mycelia they measured various resting potentials, increasing with the glucose content of the cluture medium, with top values up to 240 mV, much more than any K^+ gradient could account for. This potential is due to a greater extent to H^+ ions resulting from the energy metabolism of the cell. The more glucose energy is converted into phosphorylation of ADP, the more H^+ ions are left over. They leave the cell by diffusion - and probably also by active transport - across the membrane, thus determining a permanent cationic efflux, proportional to the uptake of glucose. This diffusion potential, added to the 30 mV of electrometric potential, corresponds to the global potential drop on the cell surface.

When the mycelium is treated with a glucolysis inhibitor, the potential drops immediately to 30 mV, while the ATP level begins to fall slowly afte some 10 seconds. If the potential depended upon an ATP-driven pump, it ought to fall as slowly as the ATP level. When the respiration is restored, within seconds the potential reappears and even overshoots its

previous level, and returns progressively while the ATP amount only slowly rises to the normal level. So the resting potential disappears when there is still some ATP, and it reappears before there is any ATP. We must conclude, that the resting potential is not the result of a ATP-driven ion pump but of an ATP-producing biochemical activity, i.e. the cell respiration.

Depending on their needs in ATP, cells may have different resting potentials. The squid axon has a low phase ATP consumption. In consequence it produces only 60 mV (30 mV boundary potential plus 30 mV H^+ flux). A striated muscle fiber is expected to produce mechanical work and must maintain a high ATP level. In fact, it produces 90 mV (30 mV phase boundary potential + 60 mV H^+ efflux). An overfed mycelium may reach 230 mV (30 mV phase boundary potential and 200 mV H^+ efflux), a value certainly nobody will try to explain by a potassium efflux potential.

But now, we face a new problem. The H-ions, in order to leave the cell, must overcome the positive potential wall built up in the membrane pores by calcium ions. Their accumulation inside the cell will lower the pH, disturb the enzymatic activities and weaken the coacervate structure. Something similar seems to happen in fact in the pre-prophase preparing the mitosis, but must be avoided in the normal cell function. There is some evidence that the expulsion of the hydrogen ions across a potential wall, but not against a concentration gradient, is realized by ATP-produced energy, and ought to be considered a true ionic pump. We shall deal with this problem after having discussed the mechanism of excitation.

The excitation (Segal, 1978; Segal et al., 1983)

Cells respond to various stimuli, so that the initial phase of excitation varies largely, but the outcome is always the same. Therefore we shall deal only with the cathodic stimulus, which plays an important role in physiology.

Due to the inequal speed of ions in the outer medium and in the cytoplasm, a cathodic current produces in the cytoplasm a polarization leading to a transitory pH drop (Fig. 5). In proteinic media it triggers a chain reaction causing a further lowering of pH and breaking of H-bridges in the proteins, resulting in new binding sites for cations, mostly H-ions. Consequently the protein becomes more electropositive (Fig. 6), until it reaches its isoelectric point. At this point the coacervate

breaks down.

The coacervate was realized by means of an endothermic dehydration. Now it is converted into a coagulate, where all molecules tend to restitute their full hydration sheet. This hydration being strongly exothermic, the water is pulled into the cell with considerable energy, and the sodium ions, firmly bound to the water, are actively sucked in. Chlorine ions, being structure breakers, do not adhers to the water and only a small proportion of them enter into the cytoplasm. The result is an active cationic inward current, producing the action potential.

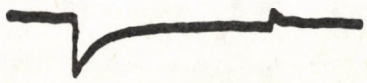

Fig. 5 The transitory pH drop under the cathode, produced by applying a 100 mV potential to a water-gelatine boundary

The potassium ions, formerly coacervate bound, pass, due to the coacervate breakdown, into compartment II, where ions are retained only by weak adhesive forces. Thus the breakdown is followed by a slow efflux of potassium, due to the concentration gradient between cytoplasm and the outside medium. But this potassium efflux is due to a passive diffusion and is furthermore reduced by the adhesive binding of potassium ions to the proteins. This efflux is necessarily weaker than the sodium influx which is actively driven by hydration forces and favored by the fact that in the outside medium sodium ions are not bound to macromolecules. Naturally, the sodium flux follows a concentration gradient of nearly the same magnitude as the outward potassium flux. But under the action of hydration forces there results a net inward cationic flux; the resulting potential shift is usually stronger than the resting potential and reverses the polarization.

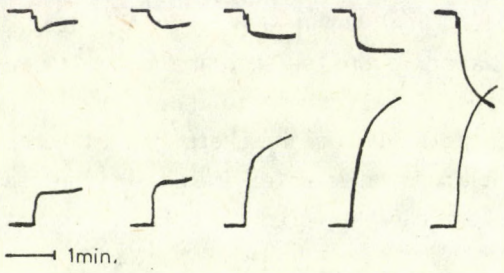

Fig. 6 The secondary reaction in protein solutions after acidification. Above - pH; below - turbidity of the protein solution (= decreasing electronegativity). The threshold of the secondary reaction is reached at the tracing 3, where a slowly decreasing pH and increasing turbidity first can be observed

The ionic fluxes display two distinct steps. After the coacervate breakdown, the driving forces are high, but so is also the membrane resistance, due to the 2 calcium ions incorporated into the pores. The inward flux detected by the "clamped potential" technique is therefore weak. The modern membrane theory explains this effect by a series of narrow gates, which are supposed to open in the membrane channels. In fact, the gating mechanism is the coacervate breakdown with subsequent hydration of the proteins; it is located in the hyaloplasm and not at all in the membrane.

When the potential shift overshoots the zero level, the $=O^{(-)}$ carbonyl group which fixes the Ca^{2+} ions inside the membrane channel shifts to the enolic form $^{(-)}C\text{-}O\text{-}H^{(+)}$. The Ca-ions in the membrane pores are liberated. They produce a short flux, detected as a "gating current" currently explained by the opening of large gates inside the channels. At this moment the strong Na^+ inward flux as well as the much weaker K^+ outward flux begin; both are strong enough to be detected by the clamped potential method.

This breakdown of the membrane resistance is a true gating effect located inside the membrane. The strong cationic influx without the corresponding anions restores the initial pH and renatures the protein molecules. They become acidic again and rebuild the coacervate state, thus repelling the excess sodium ions, with the exception of a small part of them, compensating the loss of potassium ions. Finally, during the afterpotential, these ions are exchanged by equilibrium dialysis of K_o^+ against Na_i^+ and the resting state is restored.

According to the membrane theory, the cationic activity on both sides of the membrane is the same, and the concentration gradient being nearly equal for sodium inwards and for potassium outwards, a simple opening of the membrane pores would produce nearly no effect, or rather an inverse one, due to the higher mobility of the K-ions. We explain the action potential by an active transport of Na-ions by hydration forces. The membrane theory invents a lag between the Na-influx and the K-efflux. That would require a second set of channel with narrow and large gates, but with a greater latency of response, which would be a purely arbitrary assumption.

Furthermore, the sodium channel is considered to be, after the gating, a pore large enough to let a hydrated sodium ion pass, and devoid of any unidirectional transport mechanism. Why should not a much smaller and more mobile potassium ion use the same pore to escape from the cell?

In fact even the K and Na fluxes detected by voltage clamping by the Cambridge school display perfect synchronism. In Fig. 7, published by Katz (1966), the peaks of the K and Na fluxes exactly face one another. A lag of the potassium current, often displayed in idealized pictures, is backed by no experimental evidence.

The ionic and other pumps

We shall call a mechanism a "pump" if and only if it is located either directly in the membrane or directly adjacent to it, if it either transports molecules or ions against a concentration gradient or accelerates their movement along the gradient, and uses for this purpose metabolic energy. Thus, the mechanism driving Na-ions inwards during the excitation cannot be called a pump, because its driving force results from a breakdown of coacervate in the bulk of the hyaloplasm. We are dealing

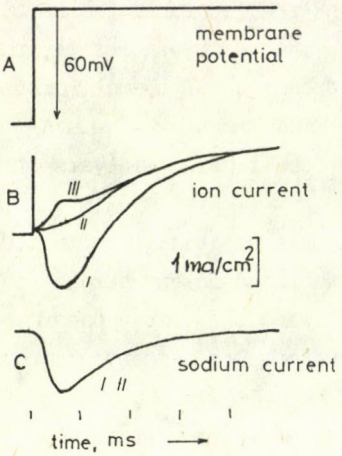

Fig. 7 Transmembrane fluxes of sodium and potassium, measured by the clamped potential technique (Katz, 1966). There is not time lag between the sodium flux (I) and the potassium flux (III)

here with an active transport but not with a membrane-located Na-pump.

The expulsion of H-ions resulting from cell respiration must be considered as real pump. Cations are pushed across a positive potential wall into the membrane pores. The ions do not move against a concentration gradient, but their efflux is accelerated by the pump. Probably a small part of this efflux is due to passive diffusion, most of it to the pumping.

We shall outline briefly a possible mechanism of its action. A more complete description is given by Segal et al (1983). The membrane ATPase, a hollow cylindrical molecule, is radially inserted into the membrane. The ATP molecules adhere to the inside rim of this drum-like structure by its adenyl group; the triphosphate tail, protruding inside the lumen, adhers to a $N\text{-}H^{(+)}$ imino group of a peptide bond by its terminal phosphate group.

When protons accumulate inside the membrane, their global positive field shifts this peptide bond from the keto towards the enol configuration, thus liberating two quanta of 1.8 eV each, amply sufficient to activate the hydrolysis. The splitting of the $P_3 \sim O$ bond results first in the formation of a steep potential gradient which, secondarily, may be used to activate endergonic chemical reactions. But in our case this gradient is used directly to push outwards the H^+ ions accumulated inside the membrane pore, across the potential barrier created by the two Ca^{++} ions in the molecular lumen.

In this moment the protons follow a concentration gradient, and energy is merely required to overcome a weak, symmetrical potential threshold. The energy expense is low, much lower than the energy production by aerobic glycolysis from which the liberation of the free protons results. Things would be different if the potential gradient measured on the cell surface resulted from an expulsion of Na^+ ions. Here a transport would be effected against the steep concentration gradient of 30:1, rising over a distance of only 70 Å, and Ling (1978) has quite correctly demonstrated that the whole energy metabolism of a cell would not be sufficient to perform such work permanently.

Clearly, with a high energy outlay such a pump could handle Na^+ ions as well as protons. Fortunately, in the resting state the concentrations of Na^+_i and Na^+_o are in equilibrium, and no expensive pumping is required. But during a peak potential, a fair amount of sodium ions enter the cell and they are likely to be expelled by the same proton pump, only with a greater expenditure of energy. We know that every spike is followed by a burst of metabolic activity over several milliseconds, quite sufficient to compensate the energy loss produced by the proton pump working as a sodium pump for a fraction of a millisecond.

There is no reason why such a pump could not handle potassium ions as well. But we have excellent experimental evidence that most of the intracellular potassium is bound to the proteins and the rest seems to be in equilibrium with the extracellular potassium. Thus a specialized potassium pump would be of little use.

The fact that certain substances inhibit the transmembrane flow of sodium while others inhibit the potassium flow is usually considered as evidence that sodium and potassium ions travel through different kinds of channels. But in a recent important study on "The origin of electrical potential in biological systems" Ohki (1985) states: "For example, tetradotoxin (TXX) and saxitoxin (STX) are inhibitors of the Na-channel of squid axon, when applied from the extracellular side. On the other hand, the specific inhibitors for K-channels are tetraethylammonium (CRA), Cs^+,etc., when applied from the intracellular phase."

Literally the same argument was used by Segal et al. (1960, p. 154). In the experiment Na^+ ions flow from the outside to the inside. Tetradoxin applied at the outer face of the membrane block this flux. K^+ flows from the inside to the outside. This flux is reduced by tetraethylammonium,

applied at the membrane's inner face. There is evidence that the two substances act at two different ends of a channel. There is absolutely no evidence that they act upon two different kinds of channels.

The same mechanism which serves as a hydrogen pump - and occasionally as a sodium pump - may activate the transmembrane flux of other substances. In most cases, special conditions must be taken into account. The calcium pump of the striated muscles may serve as an example.

In order to produce a quick and short twitch of the muscle fiber, the SR must release quickly the Ca-ions it contains and take them up just as quickly after a short time. The depolarization of the SR membrane, going along with the excitation of the sarcomer, opens the membrane pores to an outward Ca^{2+} flux. Initially, the concentration gradient is steep and the efflux is strong. Most of these ions are caught by the rapidly equilibrating calciumspecific sites on troponin. Thus the concentration of free Ca-ions in the sarcoplasm remains low, the diffusion gradient steep and the Ca^{2+} efflux rapid. It does not require any activation by an ATP-driven pump.

After a short time the membrane repolarizes and the cationic pump starts working. The same ATP energy which, in a sodium pump, is able to expel 3 Na^{+} ions against a steep concentration gradient, ought to be able to push one bivalent calcium ion inside the SR. It is not without importance that the proteins of the SR membrane differ somewhat from those of the plasma membrane, so that they conform better to the geometry of the calcium ion. But a fundamentally different pumping mechanism is not required.

Recently, Melzer et al. (1984) investigated the influence of short and long electric pulses upon the calcium uptake by the SR. With short pulses the uptake is quick and complete. With longer pulses it becomes slow and incomplete. Very likely this is not due to the shift from one pumping system to another or to the damaging effect of long depolarization upon the calcium pump. We must take into account that the sarcoplasm also bears slowly reacting calcium sites on troponin and parvalbumin. With short pulses, only the rapidly equilibrated sites are occupied, and during regeneration they release the calcium easily, thus maintaining a high calcium concentration inside the sarcoplasm. But with longer pulses an ever increasing part of the Ca^{2+} ions are captured by the slowly equilibrating sites, and then the membrane pump has to struggle against a

steep concentration gradient and displays low efficiency. Nothing happens to the pump. The functional shift is due to changing sarcoplasmic conditions.

As to other pumps, some reserve is highly required. The water-pump is nonsense, given the perfect permeability of cell membranes for water. It would be like pumping water into a sieve. Cells swell during excitation and shrink when returning to rest. This is due to binding conditions in coacervates and coagulates, in building and destroying hydration sheets. The active forces lie in the hyaloplasm and not in the membrane. Thus there is no pump.

Let us consider another example, the glucose-pump. The idea was born when membrane pores were supposed to be about 4 Å wide, so that the glucose molecule, some 10 Å wide, would require a special carrier system. Now we know that membrane pores are 20 Å wide, and in fact part of the D-glucose enters the cell so quickly that the speed of its passage cannot be measured. The same is the case for D-mannitol, a non-biological sugar. But in the following 12 seconds, the concentration of D-glucose increases progressively up to a tenfold value, while that of D-mannitol does not increase at all.

By the explanation of Jung (1973), these data are easy to understand. Radioactive D-glucose enters the cell passively through the large pores, up to an equilibrium with the outer medium. So does D-mannitol. But the glucose is rapidly converted into glucose-6-phosphate and then steps into glucogen, thus freeing the glucose compartment which is filled again from the outside. The glucose-bound radioactivity accumulates inside the cell, while the glucose concentration remains constant. As to the mannitol, it suffers no further biochemical transformation, and the level of mannitol-bound radioactivity remains at the passive diffusion equilibrium.

There is no D-glucose pump in the membrane, but there exists a glucose-transforming cytoplasmatic activity. There is no membrane pump, only an assisted diffusion.

An error was introduced by Jung et al. who used erythrocyte ghosts. The authors regarded them to be a membrane preparation, and claimed that the glucose influx was due to membrane activity. They forgot that a normal erythrocyte contains stroma proteins, which form a complete cytoplasm with big vacuoles filled by hemoglobin. In order to obtain the ghosts, one splits the Hb-molecules into 4 monomers, which escape easily through the

large membrane pores. But the stroma remains and fulfills all the usual cytoplasmic activities, including the storage of transformed glucose. Thus there is no reason to believe in a membrane-located glucose transporting pump.

Each case of alleged membrane pump must be examined separately. Pumping effects have been described, where we deal obviously with an allosteric effect of drugs or hormones, fixed on the outside of the membrane and activating an enzyme (mostly adenylcyclase) fixed on its inside face. In other cases, molecules adsorbed to the membrane are dragged inside the cell together with parts of the membrane. But such a pinocytosis is produced by the contraction of intracytoplasmic microfilaments. The active part of the mechanism does not reside in the membrane. In short, until now, with the exception of H^+, Na^+, Ca^{++} and possibly K^+, we did not encounter any membrane-located ion or molecule pump, which would withstand critical examination.

Conclusions

In this paper we have presented a phase boundary model of the living cell in its resting state as well as during the changes introduced by excitation. We integrated into this model our most recent knowledge about the static and the dynamic structure of globular proteins. We took into consideration the different forms of protein-protein interactions leading to microfilamments, membranes and coacervates, and the different forms of protein interactions with water and ions resulting from them. This model has enabled us not only to deal with the different aspects of bioelectrogenesis, but also to analyse different elementary cell functions, such as enzymatic activity, photosynthesis, mechanical work and the regulation of biochemical activity (Segal et al., 1983). In no case we encountered, substantial contradictions with experimental data, though we sometimes had to discuss apparent contradictions due to traditional ways of thinking.

The model we present in this paper is mostly based upon qualitative considerations. Its quantitative aspects (Dehmlow, 1983) will be studied in a later paper.

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LIGHT SCATTERING CHANGES DURING THE PHOTOCYCLE OF BACTERIORHODOPSIN

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SUMMARY

Spectroscopic measurements on purple membrane suspension are disturbed by the light scattering of the particles. Systematic study of the scattered light leads to the observation that scattering changes during the photocycle, and that this change depends on the flash polarization, pH, and the solvent viscosity. A theoretical model is given to explain the experimental data. The model asserts that the purple membranes are bent and, during the photocycle, the degree of bending varies due to the conformational change of the bacteriorhodopsin molecules.

INTRODUCTION

Bacteriorhodopsin (bR) is the only protein contained in the purple membrane (PM) of Halobacterium halobium. Its biological function is to pump protons out of the cell using light energy (Oesterhelt, Stoeckenius, 1973; Stoeckenius et al., 1979). The bR performs this function via a photochemical cycle whose steps were identified in the early stages of the research using visible absorption spectroscopy and flash photolysis (Stoeckenius, Lozier, 1974). Changes, taking place in the visible absorption, are caused by the alterations in the electronic structure of the retinal chromophore of the bR and the effect of perturbation by the apoprotein which absorbs light only in the UV region. Thus visible spectroscopy provides information mainly about the retinal and not the protein, although the protein must play a significant role in the photocycle of the bR.

The UV absorption and fluorescence spectroscopy are useful tools for understanding the proton pumping mechanism of bR more deeply. There has been a lot of work devoted to this area in which UV absorbance and

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fluorescence changes during the photocycle were observed (Lozier, Niederberger, 1977; Bogomolni et al., 1978; Kuschmitz, Hess, 1982; Czégé et al., 1982).

Since the PM fragments are relatively large membrane particles, the spectroscopic measurements on PM are generally distorted by light scattering: the smaller the wavelength, the higher the intensity of the scattered light (Cherry et al., 1977). It turns out that the scattered light changes during proton pumping. Because some amount of scattered light is present even when absorption kinetics are measured, light scattering changes always have to be taken into account. The present work gives a systematic study of the light scattering changes during the photocycle and also a model to interpret them. The model given to explain the changes in the scattered light can easily handle several other experimental facts which, to date had not been fully understood.

MATERIALS AND METHODS

Purple membranes were isolated from Halobacterium halobium strain JW3 according to a standard procedure (Oesterhelt, Stoeckenius, 1974). Samples were suspended in 10-50 mM KOH-CH_3COOH buffer. The sample of Fig. 5 immobilized in polyacrylamide gel was a gift from Dr. A. Dér. Measurements were performed in a 2X2 mm UV cuvette (NSG Precision Cells, Inc.). The absorbance of the samples was around 0.2 at 570 nm. A simple intelligent flash photolysis system was used to obtain experimental data (Czégé et al., 1982).

The geometry of the light scattering and absorbance measurements can be seen in Fig. 1.

The scattered light was detected at 320 nm because at this wavelength the absorbance change during the photocycle had a minimum while the scattered light intensity was acceptable.

Theoretical models were computed on a TPA 1140 computer. The tremendous amount of the computational details of the bent membrane model will be published in a forthcoming paper.

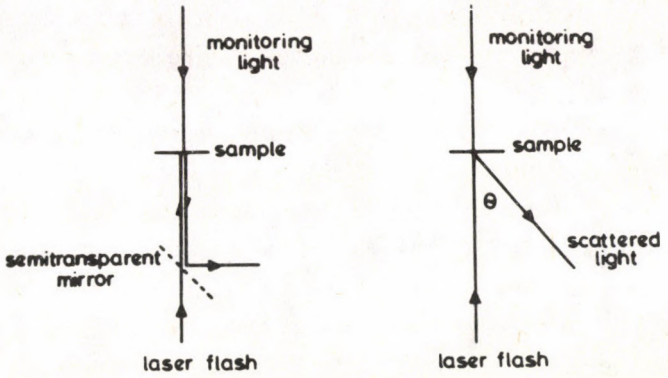


Fig. 1 Geometry of the absorbance and the light scattering measurements. The scattering light was collected in a wide range of the scattering angle, θ ($10^{o}<\theta<80^{o}$) to get enough light to increase the S/N ratio

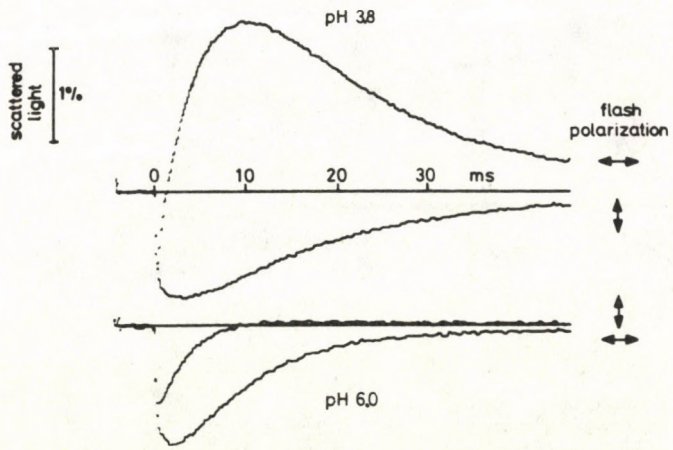


Fig. 2 Change of the scattered light during the photocycle. Note the effect of pH and the flash polarization. Temperature is 25^{o} C, λ = 320 nm

RESULTS

Fig. 2 shows the kinetics of the scattered light during the photocycle. The monitoring light (wavelength 320 nm) was not polarized because its polarization resulted in only well known changes (Kerker, 1969). The scattering plane was horizontal, the flash polarizations denoted by ↕ and ↔ were perpendicular and parallel to this plane, respectively. For the other details see the Materials and methods section. The negative spikes at the beginning of the kinetics are due to the absorbance change of the sample which has an effect on the scattered light intensity via the reabsorption. The proof of this statement can be seen in Fig. 3. Diluting the sample, the peak due to the absorbance change decreases because of the decrease of the sample concentration, while the other component remains the same on a relative scale. Moreover, the difference of the two kinetics correlates with the 410 nm absorbance decay of the photocycle (Fig. 3).

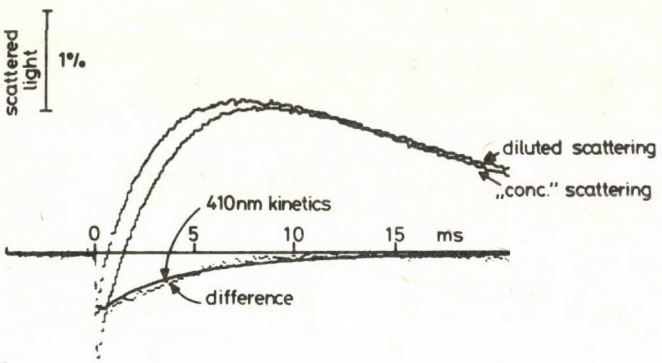


Fig. 3 Concentration dependence of the scattered light changes. Since the relative change of the light scattering cannot depend on the sample concentration thus the negative deflection must be absorbance change during the photocycle which causes change in the reabsorption of the scattered light. T=28°C, pH=3.8

As shown in Fig. 2, the kinetics of the scattered light strongly depends on pH not only in size but in signal shape, too. The result of the systematic study of the pH effect can be seen in Fig. 4a. To eliminate the part of the kinetics caused by the reabsorbance changes (which do not depend on the flash polarization), the curves represent the differences of the kinetics obtained by ↔ and ↕ flash polarization. Because the flash intensity slightly depended on the direction of the polarization, a normalizing factor of near 1 was needed at the subtraction. Fig. 4b shows the pH dependence of the amplitudes. The zero crossing is located around pH 5.1.

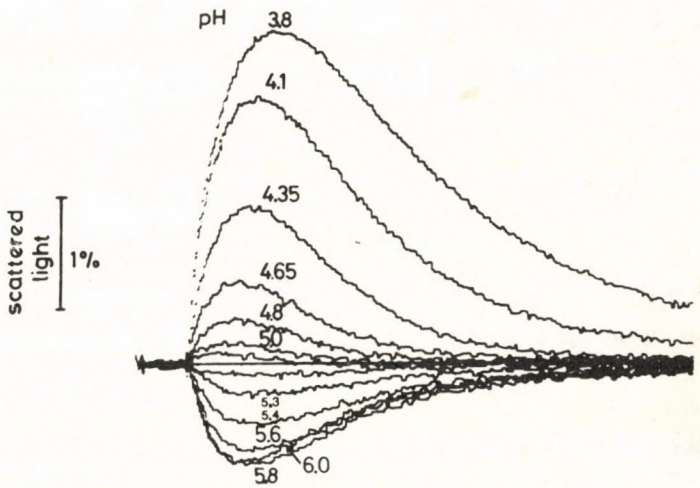


Fig. 4a The pH dependence of the scattered light kinetics. The curves are corrected (see text). T=25°C, λ=320nm

Our UV photoselection measurements published recently did not show the scattered light disturbance in the case of PM-s embedded in agar-agar gel (Czégé et al., 1982). To reveal the causes of this apparent contradiction it can be seen from Fig. 5 that in the case of the PM-s immobilized in polyacrylamide gel there is also no scattered light effect i.e., there is no difference between the kinetics gained by differently polarized flashes. Only the absorbance signal appears in the kinetics.

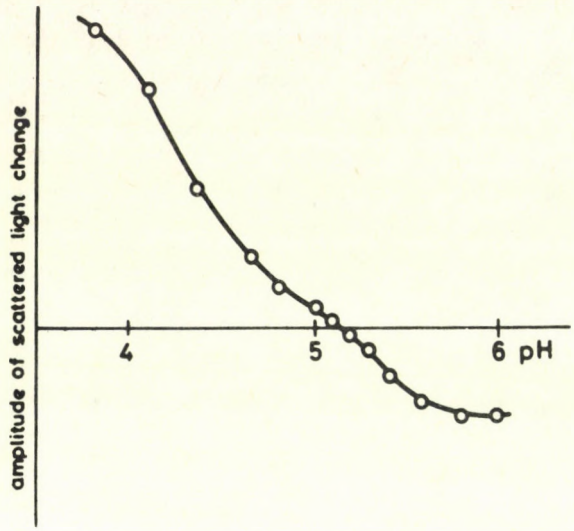


Fig. 4b The pH dependence of the maximal amplitudes of the scattered light changes

The result shown in Fig. 5 raises the question: what happens if the solvent viscosity increases continuously. The answer can be seen in Fig. 6. The viscosity was changed by adding increasing amount of sucrose to the buffer. In contrast to the effect of glycerol (Beece et al., 1981), sucrose hardly acts on the kinetics of the photocycle in accordance with its more "macroscopic" viscosity. To see better the scattered light kinetics in Fig. 6 the 410 nm absorbance signals were subtracted from the scattering kinetics. The curves in Fig. 6 show that, increasing the solvent viscosity, the relative amplitude of the scattered light change decreases and the kinetics becomes slower. Moreover, it disappears in the case of complete immobilization as it can be seen in Fig. 5.

DISCUSSION

Holding the angles fixed, light scattering depends on refraction index, particle size, and particle shape (Kerker, 1969). From the above experimental data the following conclusions can be derived:

1.) The origin of the light scattering changes during the photocycle cannot be the change of the refractive index of the PM-s or the solvent since in the immobilized sample there is no effect.

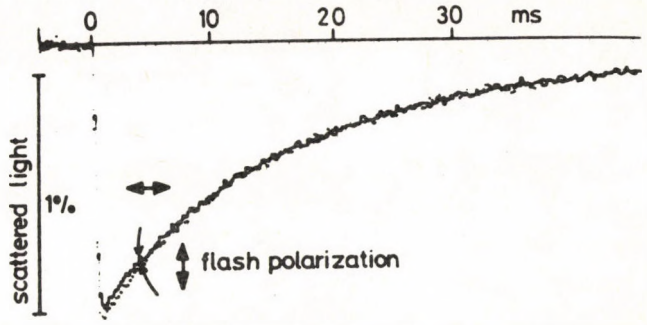


Fig. 5 Light scattering kinetics in a sample embedded in polyacrylamide gel. Only the reabsorption signal remains, the scattering changes disappear. T=28°C, pH=3.8

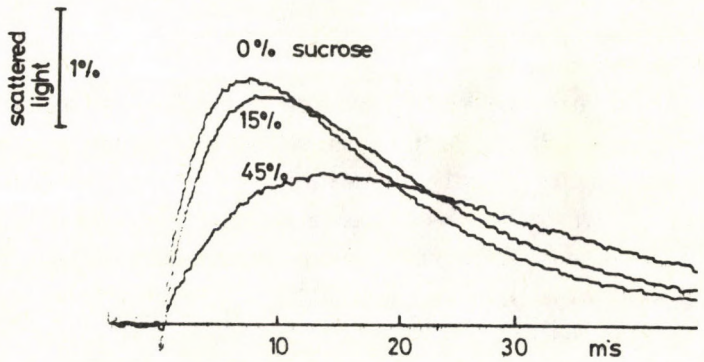


Fig. 6 Viscosity dependence of the scattering kinetics. The slow down occurs only in the scattering signal. The absorbance kinetics do not change, the curves are corrected. T=26°C, pH=3.9

2.) The same reason excludes the volume changes of the PM-s as the cause of the effect. Moreover, it would be hard to imagine the sign changes depending on pH and the flash polarization as a result of volume changes.

3.) The change of the shape of the PM-s remains as the only factor the light scattering depends on.

Based on these arguments and the experimental data the following bent membrane model (BMM) can be established. This model, which can easily interpret all the experimental data in the Results section, contains two main assertions a.) and b.).

a.) In a water suspension PM-s are bent. The bending is caused mainly by the surface charge of the PM-s which is negative on both sides but to a different degree, and at neutral pH the cytoplasmic side is more negative (Renthal, Chung, 1983; Barabás et al., 1983). This asymmetric charge distribution makes the membrane bent so that the convex side will be more negative (see Fig. 7). Since the asymmetry of the surface charge depends on the solvent pH the direction of the bending will also be pH dependent. It is known tha around pH 5 the charge distribution of the PM-s is symmetric so at this pH the membranes are nearly flat (Fig. 7).

The assertion a.) can explain the following two not fully understood data of the literature.

It is known that the angle δ between the retinal chromophore and the membrane normal slightly depends on the pH with a maximum around pH 5 (Barabás et al., 1983). Using the BMM, this result can be interpreted so that, except at pH 5, the membranes are bent. If one takes into account this fact in the evaluation of linear dichroism data then the angular dependence will disappear. The maximal bending of the membranes can also be estimated from the data published in the paper cited above.
In the case of bent membranes the reduced dichroism will be:

$$\frac{\Delta A}{A} = (3\cos^2\delta - 1)\cos(\psi/2)\ \frac{1+\cos(\psi/2)}{2} \qquad (1)$$

where ψ is the angle of the bending of the membrane (Fig. 8a). Using the Eq. 1 $\psi_{max} \sim 60\text{-}80^{\circ}$ can be calculated.

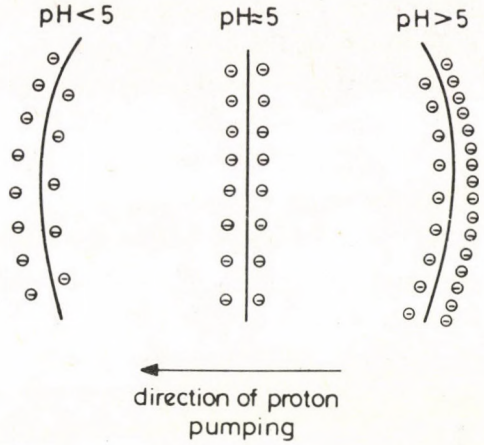
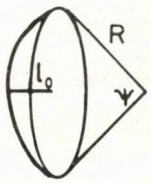


Fig. 7 The assumed bending of PM's depending on pH. The convex side is more negative, this membrane conformation is energetically more favourable



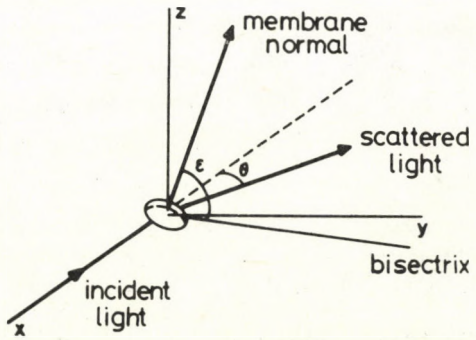


Fig. 8a Meaning of the parameters used in the scattered light considerations

The BMM explains also the direction of the proton pump in the case of the reconstituted vesicles. It is known that the inside-out vesicles are made above pH 5 while, at pH 2.5, inside-in vesicles are formed (Happe et al., 1977; Hellingwerf et al., 1978; Hellingwerf, 1979). This fact is in complete accordance with the BMM which proposes that the PM-s are bent like in intact cells below pH 5 and opposite above it.

b.) The second assertion of the BMM is that the bending of the PM-s varies during the proton pumping. The direction of this additional bending due to the photocycle has to be always the same and independent of pH. As we pointed out the permanent curvature of the PM-s changes its sign around pH 5. This means that the transient light scattering changes connected to the photocycle of bR must have opposite sign below and above pH 5.

At this point we show that by this model it is possible to explain the measured light scattering changes. Using the Rayleigh-Debye approximation (Kerker, 1969), the following form factor can be derived for a spherically bent membrane (the scattered light intensity is proportional to the form factor if the particle volume does not change):

$$P(\theta,\varepsilon) = \left|\frac{1}{\ell_o}\int_o^{\ell_o} J_o(h\rho\sin\varepsilon)\exp\{ih\ell\cos\varepsilon\}d\ell\right|^2. \qquad (2)$$

In Eq. 2 J_0 denotes the zeroth order Bessel function, $h=2k\sin(\theta/2)$, $k=2\pi/\lambda$ where λ is the wavelength of the scattered light, $\rho=\sqrt{R^2-(R-\ell)^2}$. The other parameters are given in Fig. 8a. Theoretical curves in Fig. 8b show how the intensity of the scattered light depends on the curvature of a spherical membrane holding the volume constant. The parameter of the curves is the angle between the symmetry axis of the membrane and the bisectrix of the complement of the scattering angle. The same set of curves can be seen in Fig. 8c for the case of cylindrically bent membrane.

Now, let us see qualitatively[x] how the light scattering changes in a randomly oriented membrane suspension if the membrane bending varies (see Figs 8b, 8c).

[x]Here and in the followings only those details of the more comprehensive quantitative calculations are given which are enough to anticipate the experimental facts and their theoretical explanation. The very details of the calculations will be published in a next paper.

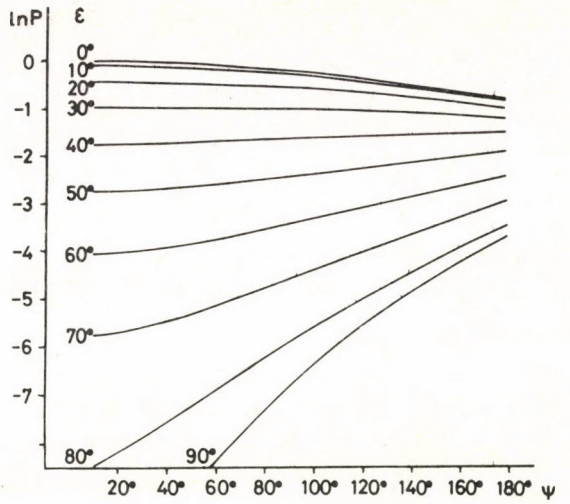


Fig. 8b The light scattering of a spherically bent membrane as a function of the bending ψ and the direction ε of its symmetry axis. The size is fixed, 25 $(\mu m)^2$, the wavelength is 320 nm. The intensity of the scattered light is proportional to the form factor P. Note the log scale

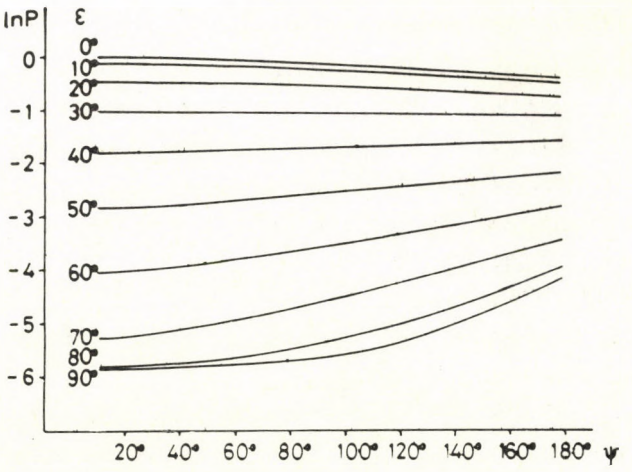


Fig. 8c The form factor curves for cylindrically bent membranes (see also Fig. 8b)

Since the retinal chromophore is near perpendicular ($\sim 70^{o}$) to the membrane normal (Heyn et al., 1977; Korenstein, Hess, 1978; Keszthelyi, 1980) by exciting the sample using $\leftrightarrow$ polarized laser flash, the membranes with big ε (near to 90^{o}) will be excited. Hence the form factor increase with increasing ψ.

In the case of the flash with $\updownarrow$ polarization among the properly oriented membranes every ε occurs. However, the scattering of the membranes with small ε is much bigger than that of PM-s with near 90^{o} ε. Thus the curves with small ε determine the scattering which means that P decreases if the ψ increases.

Numerical data which can be gained by the exact execution of the qualitative averaging outlined above verify that explanation.

From the arguments above and the experimental data in Fig. 2 it follows that the curvature of PM-s increases during the photocycle below pH 5 and decreases above it.

Now knowing the direction of the membrane bending changes let us try to find the cause of it. Since from the assertion a.) it follows that the membrane bending depends on pH, let us have a look at the effect of the local pH changes (Fig. 7) as the most natural candidate. Below pH 5 the situation is the following: Due to their positive charges, the protons released by the bR can only decrease the negative charge on the convex side. At the same time, because of the protons picked up on the concave side, the negative charge on this side of the membrane can not decrease.

Thus the local pH changes result in the decrease of the curvature. This effect is opposite in direction to the curvature change deduced from the scattering data.

Above pH 5, arguments, similar to the above ones, conclude that the origin of the changes in the bending of the PM-s during the photocycle cannot be the local pH.

Thus the only varying outer factor, pH failed to be the cause. There remains the possibility that the building blocks, the PM consists of, change their conformation during the photocycle. Since the only working element of the PM is the protein we have to conclude that the angles between the α-helices of the bR molecules change during proton pumping. The distortion is realised probably via the change of the Coulomb forces due to a charge rearrangement in the protein (Carmeli, Gutman, 1982). Similar conformational change was suggested in the literature (Bagley et

al., 1982) from the Fourier transform infrared measurements, where the estimated upper limit of the distortion was 2^{o}. However, in our case the individual angular changes of the numerous excited bR molecules within a membrane fragment sum up thus the membrane amplifies the angular distortion of the individual proteins.

The results gained by the change of the solvent viscosity can also be well explained by the BMM. Without elaborating a detailed mathematical model, let us take the parameter ψ (Fig. 8a) as a generalized coordinate. Let us suppose that the membranes bending is subject to the following forces: the frictional force $-\eta\dot{\psi}$, the bending force An(t) and the restoring force $-K(\psi-\psi_o)$. Here n(t) denotes the number of the molecules being in the state which distorts the membrane, ψ_o is the value of ψ before the photocycle, and η is proportional to the solvent viscosity. For the time course of ψ the following equation holds:

$$m\ddot{\psi} = An(t)-\eta\dot{\psi}-K(\psi-\psi_o) \qquad (3)$$

where m is the mass associated with the generalized coordinate ψ.

Before solving Eq. 3, one needs the explicite form of the time dependent force An(t). Therefore one has to know which step of the photocycle the conformational change occurs in. In our time scale, there are two candidates for this: the M form and the O form. (Using the bR-K-L-M-O-bR scheme (Beece et al., 1981) Fig. 9 shows the kinetics of the O form and that of the scattered light. (The O kinetics was measured at 640 nm). It can be seen that the rise of the scattered light change is faster which, apart from the effect of unfortunate nonlinearities, means that the conformational change occurs earlier. Therefore let us suppose that the distortion is a feature of the M form which is in accordance with the Fourier transform infrared measurements (Bagley et al., 1982).

Using routine calculations to solve the Eq. 3, one obtains the results in Fig. 10. Taking into account that Eq. 3 is only a very simplified model of the motion of the PM-s, the similarity between Fig. 6 and Fig. 10 seems to be convincing.

Finally, the following notices have to be considered. The light scattering changes discussed above occur at all wavelengths in the 240-750 nm range and have the same character as at 320 nm. This means that when measuring absorption kinetics or performing photoselection measurements on

the water suspension of PM one always has to take into account to some extent disturbances due to the light scattering effects.

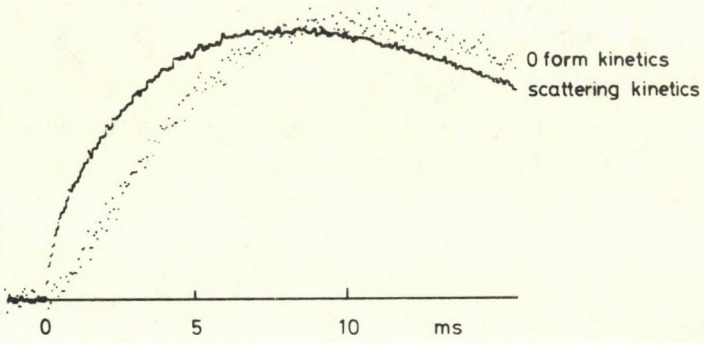


Fig. 9 Relative kinetics of the O-form and the light scattering. The M absorption kinetics is subtracted from both curves (See Fig. 3). The amplitude of the correction is the first negative deflection jump of the kinetics. The rise of the scattering is faster. T=22.5°C, pH=3.9

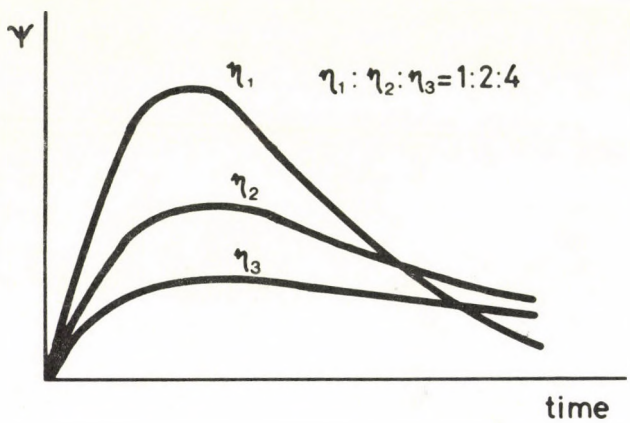


Fig. 10 Kinetics gained by solving the Eq. 3. $\eta/m=3.5$, $K/m=3$, $A/m=\exp(-t/\tau)$, where τ is the M form decay time

However, using nonpolarized flash to excite the photocycle the light scattering transient may considerably decrease since the two components of the scattering kinetics are opposite in sign (Fig. 2).

The results described in this paper strongly substantiate conformational changes in the working bacteriorhodopsin. They also provide a method to amplify and therefore make accessible these small molecular distortions for a relatively simple optical experimental technique.

Acknowledgements

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BENT MEMBRANE MODEL OF THE PURPLE MEMBRANE. THEORETICAL DETAILS AND FURTHER EXPERIMENTAL DATA

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SUMMARY

Theoretical treatment of the bent membrane model of purple membrane is given. Beside the derivation of the formulae, the results of the numerical calculations concerning the angle dependence of the light scattering changes are presented. The paper contains also experimental data on the scattering changes during the photocycle at several scattering angles, which are in good accordance with the theory.

INTRODUCTION

Light scattering measurements on working purple membrane suspension (Czégé, 1985) led to the conclusion that the purple membrane fragments are bent depending on pH, namely they are bent like in the cell membrane below pH 5 and the bending is opposite above pH 5. During the photocycle of the protein this bending changes, which causes a transient in the light scattering of the purple membrane fragments if one uses polarized light pulse to excite the photocycle. The polarized actinic light results in an anisotropically excited population of the purple membranes which makes possible to detect the small changes in the bending via light scattering measurements.

In this paper the theoretical details of the calculations, which describe the light scattering changes during the photocycle of the purple membranes, and new experimental data on the scattering angle dependece are reported.

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MATERIALS AND METHODS

Purple membrane fragments were isolated from Halobacterium halobium strain JW3 using standard procedure (Oesterhelt, Stoeckenius, 1974). Light scattering measurements were performed in a 0.2 mm flat cuvette and an intelligent flash photolysis system (Czégé et al., 1982) was used to collect and treat experimental data. Other experimental details are described elsewhere (Czégé, 1985)

Theoretical calculations were performed on a TPA-1140 computer.

RESULTS AND DISCUSSION

a) Light scattering of cylindrically bent membrane

In the following we approximate the purple membrane with a square which is bent cylindrically like in Fig. 1. Doing so, it is much more difficult to see qualitatively the consequences of the photoselection as it was in the case of the spherical approximation published in our previous paper (Czégé, 1985). Nevertheless, we prefer the cylindrically bent membranes, because in that case the way of bending (and its change, too) is quite trivial, while the spherical approximation - as it can be easily seen - requires a rearrangement of the protein trimers within the membrane during the change of the membrane bending and such rearrangement is not likely to occur because of the rigidity of the purple membranes.

To calculate the light scattering we use the Rayleigh-Debye approximation (Kerker, 1969) because the membrane is very thin (5 nm) as com pared to the wavelength of the scattered light (λ = 320 nm). Therefore

$$I_{\perp} \sim V^2 P(\theta), \qquad I_{\parallel} \sim V^2 P(\theta) \cos^2\theta$$

where $I_{\perp}$ and $I_{\parallel}$ are the scattered intensities when the incident light is polarized perpendicularly and in parallel to the scattering plane, V is the volume of the scattering particle, θ is the scattering angle and $P(\theta)$ is the so called form factor:

$$P(\theta) = \frac{1}{V^2} \left| \int_V e^{ihs} dV \right|^2$$

where $h = (4\pi/\lambda)\sin(\theta/2)$, λ is the wavelength of the scattered light and s is the coordinate of the projection of an arbitrary point of the membrane

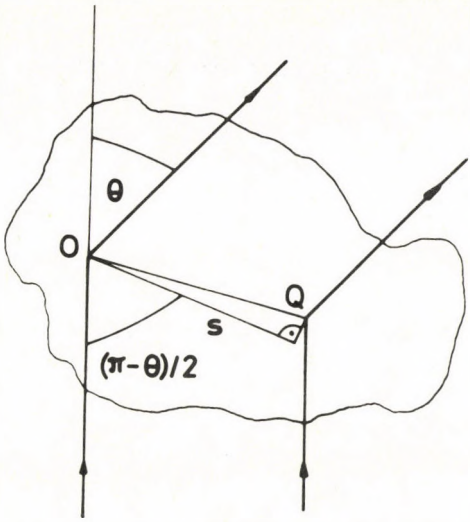
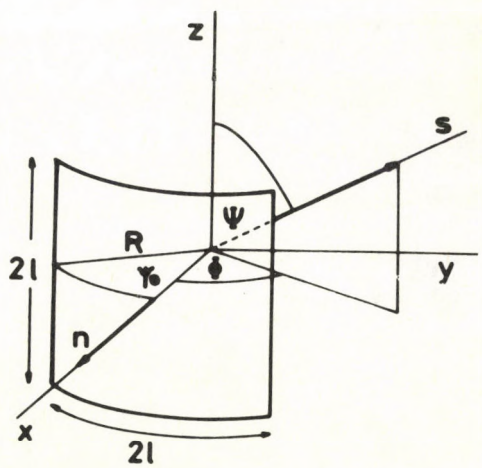


Fig. 1 Geometry of the light scattering (a) and the bent membrane (b) for the calculation of the form factor



to the bisectrix of the complement of θ (Fig. 1). Here and in the following we assume that the volume does not vary essentially during the transient change of the membrane shape.

After elementary calculations we obtain for the form factor:

$$P(\theta) = \frac{\sin(h\ell\cos\Psi)}{h\ell\cos\Psi} \left| \frac{1}{2\psi_o} \int_{-\psi_o}^{\psi_o} \exp\{ihR\sin\Psi\cos(\phi-\Phi)\}d\phi \right|^2$$

where Ψ is the angle between the axis of the cylinder and the line s, $\cos\Phi = \cos\varepsilon / \sin\Psi$ where ε is the angle between the normal, n corresponding to the centre of the membrane and the line s, $2\psi_o$ and R are the angle and the radius of the membrane bending, respectively.

Now we have to determine the excitation and the bending (during the photocycle) for differently oriented purple membranes and to compute the average of the corresponding form factors.

Hence, the next step is the calculation of the excitation of a single purple membrane which is excited by a polarized light pulse.

b) The measure of the excitation of a purple membrane

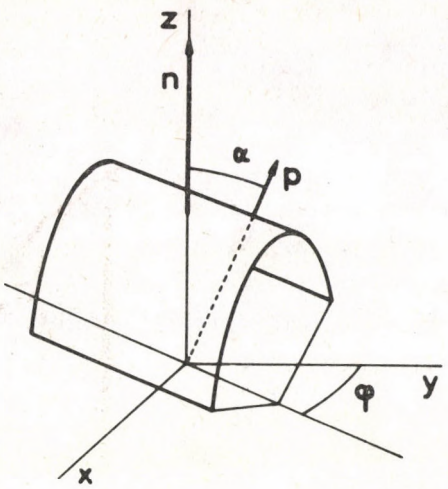
It is well known that the probability w of the excitation of a chromophore with light is proportional to the square of the corresponding transition dipole moment, μ and the square of the cosine of the angle ϑ between the direction of the light polarization and that of the transition dipole moment:

$$w \sim \mu^2\cos^2\theta$$

Because in the following only the angle θ will change, we can take the $\cos^2\theta$ as the measure of excitation:

$$e = \cos^2\theta .$$

Let us place a bent membrane into a coordinate system as it can be seen in Fig. 2. Bacteriorhodopsin molecules and the retinal chromophores which are essential with respect to the excitation form trimers in the membrane. It is plausible to suppose that the change of the membrane bending starts from the bending existing before the excitation and the direction of the axis of the cylinder does not change. Our other assumption is that the change of the bending is proportional to the average excitation of a retinal in the membrane. Therefore in the next step we have to calculate the average excitation of a retinal chromophore in a trimer.



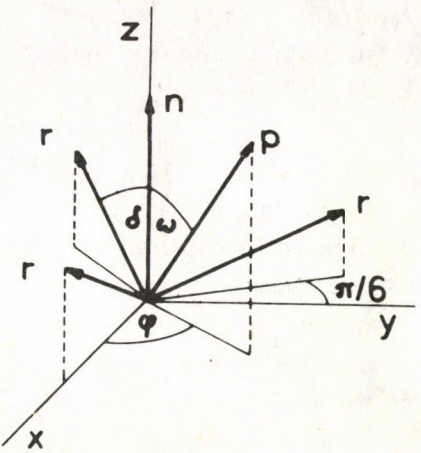


Fig. 2 (left) Geometry for the calculation of the excitation of the bent membrane
Fig. 3 (right) Average excitation of a single bacteriorhodopsin trimer

For the trimer shown in Fig. 3 we easily obtain the equation:

$$e = \cos^2\delta\cos^2\omega + \frac{1}{2}\sin^2\delta\sin^2\omega = \frac{1}{2}\sin^2\delta + \frac{1}{2}(2-3\sin^2\delta)\cos^2\omega$$

where δ is the angle between the retinal transition dipole and the trimer "normal", n and ω is the angle between the light polarization, p and n.

Using that result, let us determine the average excitation of a retinal chromophore on an arbitrary membrane. (Fig. 2) This needs to average the $\cos^2\omega$ over the membrane. It can be seen from the figure that

$$\cos^2\omega = (p,n)^2 = \sin^2\alpha\sin^2\phi\sin^2\psi + 2\sin\alpha\cos\alpha\sin\phi\sin\psi\cos\psi + \cos^2\alpha\cos^2\psi$$

The desired mean value will then be

$$\langle\cos^2\omega\rangle = \frac{1}{2\psi_o}\int_{-\psi_o}^{\psi_o}\cos^2\omega\,d\psi = (\sin^2\alpha\sin^2\phi - \cos^2\alpha)\langle\sin^2\psi\rangle + \cos^2\alpha.$$

Straight forward calculation leads to the following result:

$$e = \frac{1}{2}\sin^2\delta + \frac{1}{4}(2-3\sin^2\delta)\left|\sin^2\alpha\sin^2\phi + \cos^2\alpha - \frac{\sin(2\psi_o)}{2\psi_o}(\sin^2\alpha\sin^2\phi - \cos^2\alpha)\right|$$

where α is the angle between the central membrane normal and the polarization of the actinic light, and ϕ is the angle between the axis of the cylinder and the p,n plane.

If we suppose that the change of the membrane bending is originated by the distortion of the excited proteins then we can take $\Delta\psi_o$ to be proportional to the excitation e:

$$\Delta\psi_o = Ee.$$

Since the averaging of the form factor for a selectively excited membrane population can be executed only by numerically, we have to estimate ψ_o and E, and the membrane size which mainly determine the light scattering. All these are necessary because the numerical calculations needed several hours computer time of the available TPA-1140 computer even for a single set of the parameters.

c) Estimate of the basic bending

We can get information about the basic (without excitation) bending of the membrane supposing that the weak pH dependence of the orientation of the retinal chromophore (Barabás et al., 1983) is the consequence of the membrane bending and not the retinal deflection within the membrane. This assumption requires the correction of the formula for the reduced dichroism used in the literature.

Now, let us determine this correction for cylindrically bent membranes. When oriented by electrical field, the absorption of the purple membrane suspension, measured by polarized light, changes.

Let $A_{\parallel}$ denote the absorbance, measured by light polarized parallel to the orienting field, and let A be the absorbance of the random sample. In the above-mentioned paper the authors calculated the angle of the chromophore using the formula:

$$\frac{A_{\parallel} - A}{A} = 3\cos^2\delta - 1.$$

Since the absorbtion is proportional to the excitation, e used in the present paper, thus

$$A_{\parallel} \sim e_{\parallel} = \frac{1}{2}\sin^2\delta + \frac{1}{4}(2-3\sin^2\delta)\left(1+\frac{\sin(2\psi_o)}{2\psi_o}\right).$$

(In that case $\cos^2\alpha = 1$ and $\sin^2\alpha = 0$.)

For the random sample e=1/3, hence

$$\frac{A_{\parallel} - A}{A} = 3e_{\parallel} - 1 = (2-3\sin^2\delta)\frac{1+3\frac{\sin(2\psi_o)}{2\psi_o}}{4} = (3\cos^2\delta - 1)\left(1+3\frac{\sin(2\psi_o)}{2\psi_o}\right)/4.$$

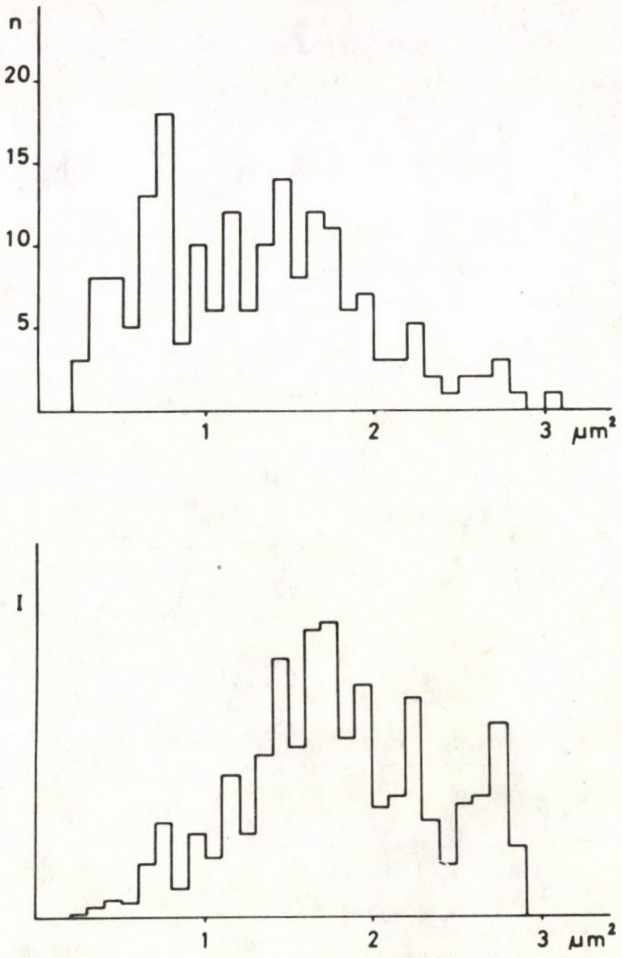


Fig. 4 Size distribution (a) and the light scattering intensity distribution (b) in the membrane suspension

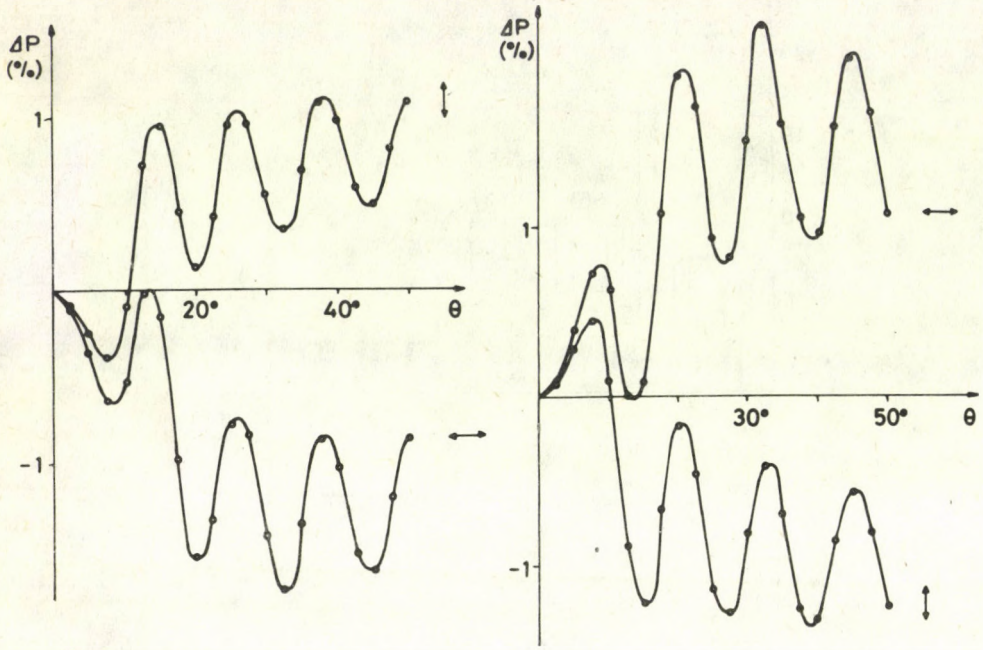


Fig. 5 Theoretical dependence of the form factor on the scattering angle θ: a) E<0, b) E>0. Size= 1.7 $(\mu m)^2$, $\psi_o = 37^o$

Consequently for cylindrically bent membrane the correction factor is $(1+3\frac{\sin(2\psi_o)}{2\psi_o})/4$.
Provided, tha this factor explains the 2-3° difference in the orientation of the retinals between pH5 and pH6, we get for the membrane bending the value 20-30° concerning the membranes of 0.5 μm linear extension.

d) The characteristic membrane size

To determine the characteristic size from the membrane population with respect to the light scattering intensity we measured the area distribution of the fragments electronmicroscopically (Fig. 4). Since the scattered intensity depends on the square of the particle size beside the form factor, thus the distribution has to be weighted with the size square. This way the size giving the maximal light intensity was 1.7 $(\mu m)^2$.

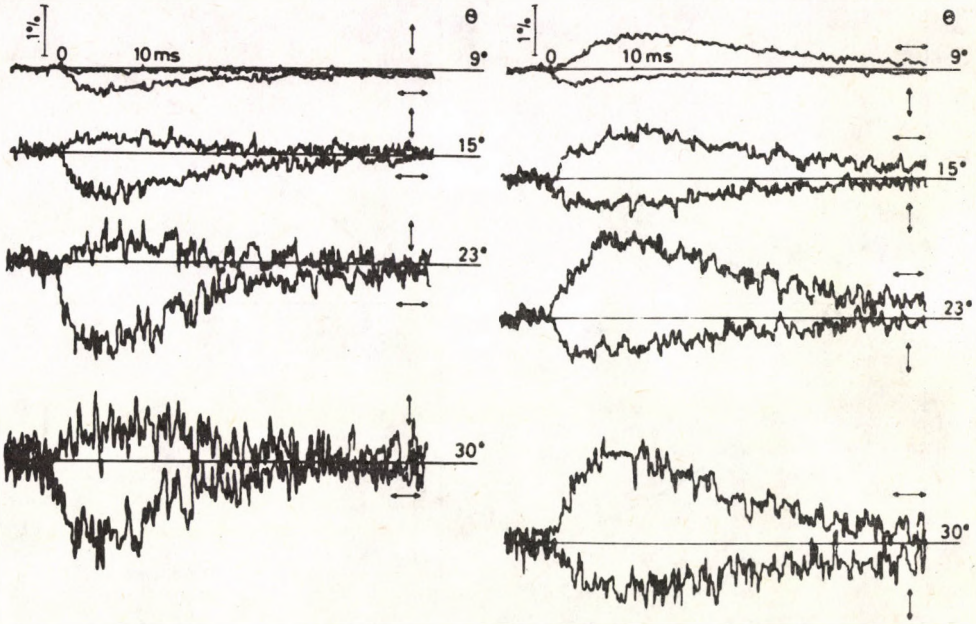


Fig. 6 Set of experimental kinetics of the light scattering during the photocycle of bacteriorhodopsin as a function of the scattering angle: a) pH=6, b) pH=3.8

e) Results of the numerical calculations

Taking into account the above-mentioned facts, the typical values of the necessary parameters are the followings:

$$\delta = 70^{o}$$

$$\psi = 0.65 = 37^{o}$$

$$E^{o} = \pm 15^{o}.$$

The positive and negative value of E correspond to the increase and the decrease (respectively) of the bending during the photocycle. This value of E gives around 1% change in the light scattering, which was the result of the experiments reported in the literature (Czégé, 1985).

These parameters result in the computed curves in Fig. 5. The symbols $\leftrightarrow$ and $\updownarrow$ mean the cases of the actinic light polarization parallel and perpendicular to the scattering plane. E>0 and E<0 correspond to pH 5 and pH 5, respectively (Czégé, 1985).

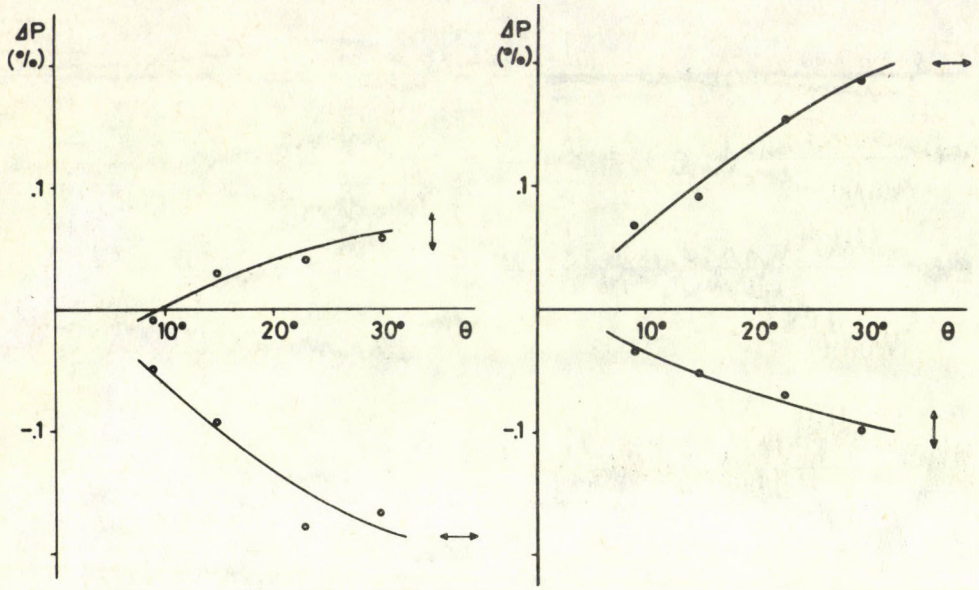


Fig. 7 Maximal elongations of the curves in Fig. 6:
a) pH=6, b) pH=3.8

In the next point we find experimental data for several scattering angles.

f) Scattering angle dependence of the transient light scattering.

In Fig. 6 we can see the kinetics of the transient light scattering during the photocycle of the purple membrane, measured at several scattering angles. Giving the angles we took the refractive index of the sample to be equal to that of the water. At bigger scattering angles it was impossible to measure kinetics because of the very small intensities. The opening of the detector was $\pm 8^{o}$. The original scattering kinetics were corrected by the absorbance kinetics measured at 410 nm in order to eliminate the reabsorbance peak. The experimental proof of that procedure can be found in an earlier paper (Czégé, 1985).

Fig. 7 shows the amplitudes of the transients as a function of the scattering angle. Comparing to the theoretical curves, we can see that experiments verify the computed data in the following sense: The theo-

retical curves in Fig. 5 show an oscillation which is the result of an interference, and the frequency of which depends on the size of the particle: the bigger the particle the higher the frequency. Hence, comparing Figs. 5 and 7, one has to neglect the oscillations. At the same time, apart from the oscillations the relative amplitudes of the curves in Fig. 5 excellently fit those of the experimental curves in Fig. 7.

Acknowledgements

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ELIMINATION OF UV-RESISTANCE INDUCING EFFECT OF R46 R FACTOR BY 5-FLUROURACIL

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SUMMARY

R-factor curing capacity of 5-fluorouracil (5-FU) was studied. Well detectable increase in UV-resistance was found in E.coli K12 AB1157 strain and its recB$^-$, recC$^-$, recF$^-$ mutants harbouring R46 R-factor. After treatment with 5-FU, these strains lost not only the antibiotic resistance coded for R46 R-factor but their UV-radioresistance, as well.

INTRODUCTION

Some R plasmids confer resistance to a number of chemical agents, metal ions and UV-light, besides the protection against antibiotics (Ericson, 1969; Foster, 1983; Sutton, Jacoby, 1978).

The N group R plasmid R46 is among those known to protect against UV damage. The size of R46 is 51.7 kilobases pairs. It codes for resistance to ampicillin, tetracyclin, sulfonamide, and streptomycin (MacPhee, 1973; Mortelmans, Stocker, 1976; Tweats et al., 1976).

The R46 R factor became the favorite object of radiobiological studies because of its property to give protection against UV damage. Later one of its derivatives with smaller molecular weight was expected to be more easily transferable.

Mortelmans and Stocker (1976) produced the pKM101 plasmid from R46 by P22 transduction and serial transfer. Its capacity to mediate UV protection and mutagenesis remained, just like the resistance to ampicillin, however, its resistance to the other three antibiotics mentioned above had been lost.

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In our experiments, presented in this paper, we made an attempt at eliminate the resistance to antibiotics due to the presence of R46 R plasmid by 5-fluorouracil preserving the genes responsible for UV protection at the same time.

MATERIALS AND METHODS

Bacteria

E.coli K12 strains used as recipients were: E.coli K12 AB1157 (which is wild type with respect to both excision and recombination repair) and its rec$^-$ derivatives (recB$^-$, recC$^-$, recF$^-$).

E. coli J5-3(R46) strain was used as donor.

Bacterial media: Nutrient broth was 8 g/liter nutrient medium with 5 g/liter NaCl. Nutrient agar plates contained nutrient medium with 15 g/liter agar (Difco). Minimal glucose plates contained minimal medium (Tweats et al., 1976) with 20 g/liter agar. Top agar was 6 g/liter agar with 5 g/liter NaCl.

R-factor elimination: Exponential-phase cultures were grown to approximately 2×10^8 organisms/ml in suitably supplemented Davis-Mingioli media (DM) (Davis, Mingioli, 1950). Cultures (0.2 ml) were added to 9.8 ml of fully supplemented DM containing 1 g/liter of 5-fluorouracil and incubated at 37 ^oC. After 24 hr of incubation samples were taken, diluted in nutrient broth, and plated on nutrient agar. After overnight incubation the number of colonies was counted on each plate and suitable clones were replicate-plated to test for R-factor retention.

Irradiation: Exponential-phase cultures were washed and resuspended to 10^8 viable organisms/ml at 20 ^oC on Davis-Mingioli salt solution which lacked the carbon source (DM base). Two milliliters of this suspension were transferred to a 5-cm diameter glass Petri dishes and irradiated.

Ultraviolet irradiation was made while stirring the suspension continuously under BLF model low pressure mercury lamp. The position of the lamp was either 18 cm above the sample, giving a dose rate of 5.2×10^{-6} J/mm^2/s or 60 cm above the sample (dose rate of 1.4×10^{-6} J/mm^2/s). Doses were determined using a model Black-Ray UV meter Nr. J 225.

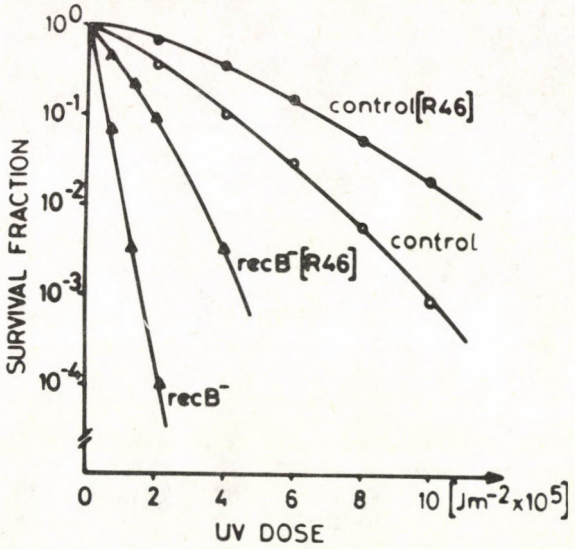

Fig. 1 Effect of R46 on the survival of E.coli K12 AB1157 and that of recB⁻ mutant after UV irradiation

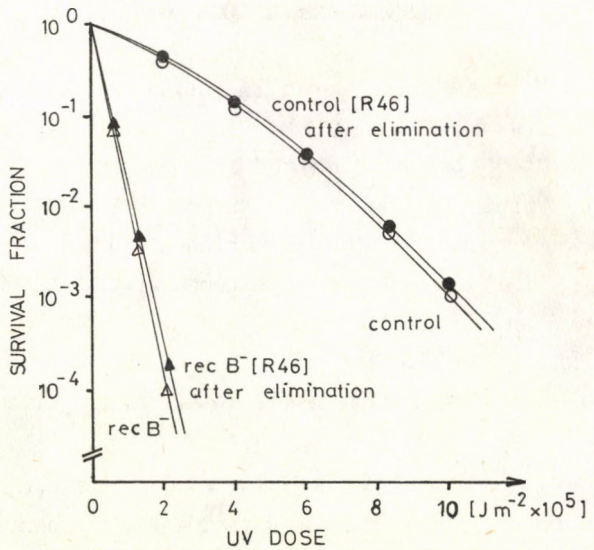

Fig. 2 Effect of R46 on the survival of E.coli K12 AB1157 and that of recB⁻ cured by 5-fluoro-uracil

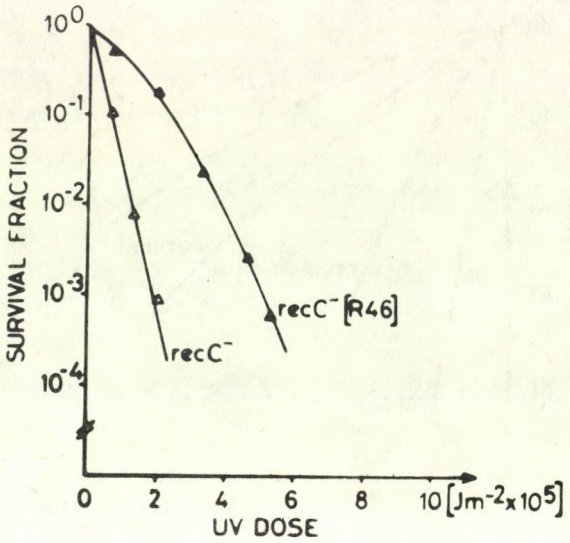

Fig. 3 Effect of R46 on the survival of recC⁻ mutant after UV irradiation

RESULTS AND DISCUSSION

The effects of R46 R-factor on UV radiation sensitivity are shown as dose-response curves in Figs. 1,2,3,4,5 and 6. As it is shown in Fig. 1, the presence of R46 R factor resulted in significant increases in the radioresistance values of the control E.coli K12 AB1157 strain and the recB⁻ mutant. Fig. 2 shows that the cured clones had lost the acquired UV-radioresistance. Similar results were obtained with recC⁻ (Figs. 3 and 4) and recF⁻ (Figs. 5 and 6) mutants.

According to our experimental results R46 R factor gave a significant protection against UV radiation damage in all cases (E. coli K12 AB1157 strain, the recB⁻, recC⁻ and recF⁻ mutants).

Further, we wanted to know whether an R factor curing agent could also eliminate the ability of the R46 R factor to increase UV-resistance. As in the previous experiments 5-FU proved to be an active eliminator of R46 from E.coli K12 J-53 strain (Pinney, Smith, 1973), the curing effect of 5-FU was also tested. Only the clones that had lost their R46-coded

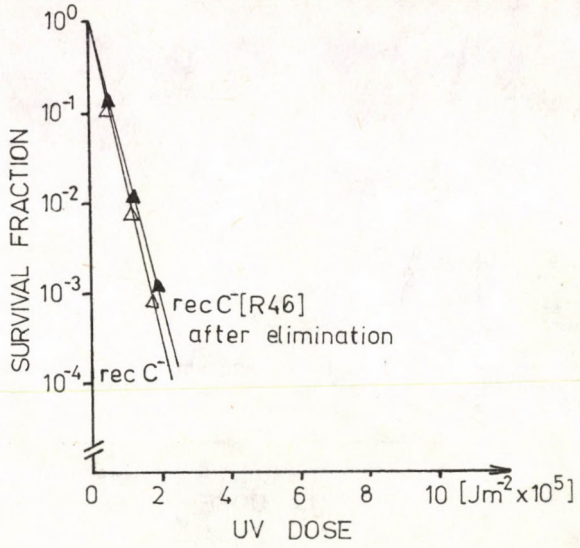

Fig. 4 Effect of R46 on the survival of recC⁻ after UV irradiation and recC⁻ cured by 5-fluorouracil

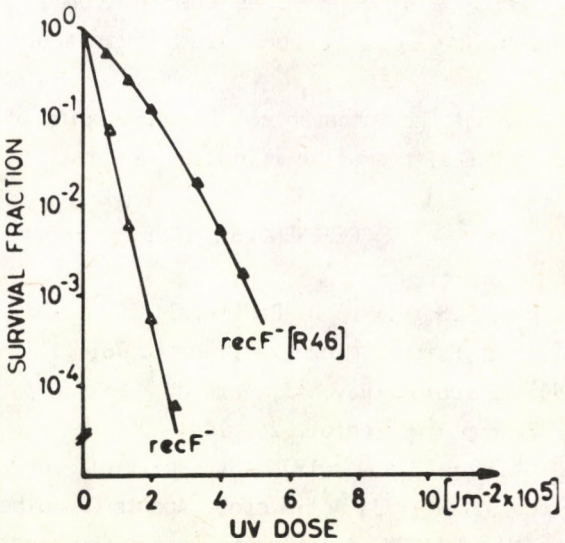

Fig. 5 Effect of R46 on the survival of recF⁻ mutant after UV irradiation

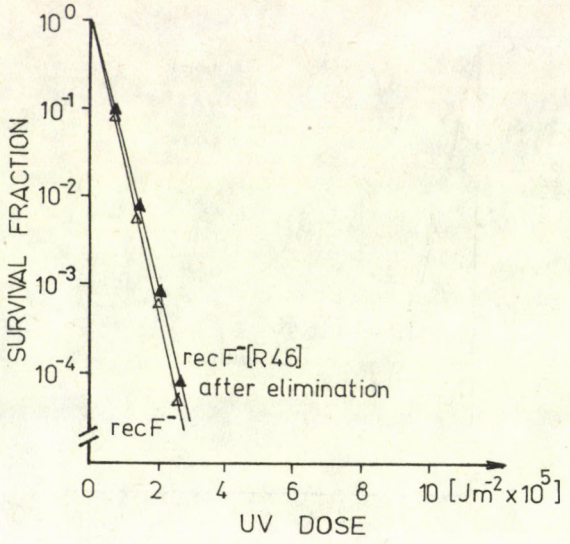

Fig. 6 Effect of R46 on the survival of recF⁻ after UV irradiation and recF⁻ cured by 5-fluorouracil

resistance to all the four antibiotics (ampicillin, tetracyclin, sulfonamide, and streptomycin) were further tested for radioresistance. The cured recB⁻, recC⁻ and recF⁻ lines lost not only the antibiotic resistance coded by R46 R factor but also their acquired UV-radioresistance.

Thus, 5-FU proved to be inconvenient for developing of UV-resistance coding derivative of R46 with smaller molecular weight.

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