Biochimica et Biophysica Acta, 462 (1977) 380–389

BBA 47388

INTERACTION OF A SYNTHETIC POLYANION WITH RAT LIVER MITOCHONDRIA

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(Received May 10th, 1977)

SUMMARY

The effect of a polyanion (a copolymer of methacrylate, malaete and styrene in a 1 : 2 : 3 proportion with an average molecular weight of 10 000) on respiration, ATPase activity and ADP/ATP exchange activity of rat liver mitochondria and submitochondrial particles has been studied.

The polyanion (at 17–150 μg/ml concentration, 100 μg polyanion corresponding to 0.83 μequiv. of carboxylic groups) inhibits the oxidation of succinate and NAD-linked substrates in state 3 in a concentration-dependent manner. The extent of this inhibition can be decreased by elevating the concentration of ADP. State 4 respiration is not affected by the polyanion. It has also a slight inhibitory effect on the oxidation of the above mentioned substrates in the uncoupled state (a maximum inhibition of 37 % at 166 μg/ml polyanion concentration), which is unaffected by ADP. The strong inhibition of state 3 respiration can be relieved by 2,4-dinitrophenol to the low level observed in the uncoupled state. Ascorbate+TMPD oxidation is slightly inhibited in state 3, while it is not inhibited at all in the uncoupled state.

The polyanion, depending on its concentration, strongly inhibits also the DNP-activated ATPase activity of mitochondria (50 % inhibition at 40 μg/ml polyanion concentration).

The ATPase activity of sonic submitochondrial particles is also inhibited. However, this inhibition is incomplete (reaching a maximum of 65 %) and higher concentrations of the polyanion are required than to inhibit the ATPase activity of intact mitochondria.

The polyanion inhibits the ADP/ATP translocator activity of mitochondria, measured by the "back exchange" of [2-^{3}H]ADP. After a short preincubation of the mitochondria with the polyanion, the concentration dependence of the inhibition by the polyanion corresponds to that of the DNP-activated ATPase activity of intact mitochondria.

It is concluded that, in intact mitochondria, the polyanion has at least a dual effect, i.e. it partially inhibits the respiratory chain between cytochrome *b* and cyto-

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DNP, 2,4-dinitrophenol; EGTA, ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid.

chrome *c*, and strongly oxidative phosphorylation by blocking the ADP/ATP translocator.

INTRODUCTION

Reversible interactions between polycations and the mitochondrial cytochrome system has been described [1–4] and an alteration of the anion permeability of the mitochondrial inner membrane by protamine – a polycation – has been also supposed [5]. In recent papers, Ogata and Kondo [6, 7] have suggested that a polyanion, dextrane sulphate inhibits competitively the translocation of ADP across the inner membrane of mitochondria. Luciani [8] has concluded that it is the dicarboxylate translocator which is inhibited by tannic acid, also a polyanion. The latter reports indicate a specific interaction between polyanions of different structure and the outer surface of the mitochondrial inner membrane.

Recently, we started a systematic research to correlate the effects of polyanions of different structure (i.e. composition, charge density, hydrophobicity, etc.) and of different molecular weights with their effect of mitochondrial functions. It was anticipated that these different polyanions might provide important information about the localization of functions within the inner membrane and also about the permeability properties of the outer membrane.

In this paper, the effects of one of the polyanions investigated, a copolymer of methacrylate/maleate/styrene in a 1 : 2 : 3 ratio having an average molecular weight of 10 000 on rat liver mitochondria are described. It is shown that it inhibits strongly the ADP/ATP translocator and also slightly the respiratory chain. In addition, a partial inhibition of the ATPase activity of sonic submitochondrial particles is also demonstrated. Parts of the present work have already been published in abstract form [9, 10].

MATERIALS AND METHODS

Rat liver mitochondria were prepared essentially according to Schneider [11] in a medium containing 250 mM sucrose, 1 mM EGTA and 4 mM Tris · HCl buffer (pH 7.2), using a teflon-glass homogenizer driven by hand and washed twice. The final pellet was resuspended in the same medium.

Submitochondrial particles were prepared according to Gregg [12] with minor modifications. The stock suspension of mitochondria (containing about 60 mg mitochondrial protein/ml) was diluted with 10 vols. of 10 mM Tris · HCl buffer (pH 7.2) and sonicated successively for 15-s bursts with 30-s breaks in an MSE 100 W sonicator set at 7 with 22 kc using ice-cooling. Intact mitochondria were removed at 15 000 $\times g$ for 20 min and the supernatant fraction was centrifuged at 144 000 $\times g$ for 30 min. The final pellet was resuspended in the medium used for the preparation of mitochondria.

Oxygen uptake was measured with a Clark-type oxygen electrode at 37 °C in a 3 ml volume in a medium containing 80 mM KCl, 1 mM EGTA, 5 mM Tris/potassium phosphate (pH 7.2), 20 mM Tris · HCl buffer (pH 7.2) using about 2 mg mitochondrial protein. Other experimental details are given in the legends to the figures and tables.

ATPase activity was measured with some modification of the method described

by Weiner and Lardy [13] in a 1.5 ml volume. The standard reaction medium contained 80 mM KCl, 1 mM EGTA, 20 mM Tris · HCl buffer (pH 7.2), 6 mM ATP and 200 μM DNP. When ATPase activity of submitochondrial particles was measured, $MgCl_2$ (4 mM) was also present in the incubation medium. The reaction was started by the addition of mitochondrial suspension (or submitochondrial preparation) containing about 1.5 mg (0.4 mg) protein to tubes at 37 °C. After a 3 min incubation at this temperature, the reaction was stopped by the addition of 1.5 ml ice cold 10 % trichloroacetic acid and the precipitate centrifuged. Inorganic phosphate was determined in the supernatant according to Fiske and SubbaRow [14]. Under these conditions about 20 % of the added ATP was hydrolyzed by the mitochondria and the reaction rate was linear with time (at least for 6 min). Further experimental details are given in the legend to the figures.

Adenine nucleotide translocator activity was measured essentially according to the "back exchange" method of Pfaff and Klingenberg [15] using [2-^{3}H]ADP as substrate and carboxyatractyloside to stop the reaction [16]. Mitochondria (containing about 100 mg protein) were labelled with [2-^{3}H]ADP by incubating them in a medium containing 110 mM KCl, 20 mM Tris · HCl buffer (pH 7.2), 1 mM EGTA and 133 μM ADP containing 6 μCi [2-^{3}H]ADP at 0 °C for 30 min. The mitochondria were washed three times by centrifugation to remove external radioactivity and suspended in the medium used for preparation of mitochondria. For exchange measurements, mitochondria were incubated in 2.2 ml volume in a medium containing 110 mM KCl, 20 mM Tris · HCl buffer (pH 7.2), 1 mM EGTA and 230 μM unlabelled ADP at 0 °C. The reaction was usually started by addition of [2-^{3}H]ADP-loaded mitochondria (containing about 6 mg protein). When the effect of preincubation of the polyanion with the mitochondria was studied, the reaction was started by the addition of unlabelled ADP. After 20 or 30 s, the reaction was terminated by the addition of 25 μM carboxyatractyloside. Mitochondria were separated quickly by centrifugation (20 000 $\times g$ for 5 min). The radioactivity of the supernatant was measured with a Beckman LS 355 type liquid scintillation spectrometer using a scintillation cocktail containing 0.8 % PPO, 0.01 % POPOP and 5.5 % naphtalene in a mixture of dioxane/methylcellosolve/toluene (10 : 2 : 1, by vol.). Correction was made for the leakage of labelled ADP. The rate of exchange was found to be linear with time within 1 min under these conditions. The effect of polyanion on the translocator is expressed in percent of the activity of the appropriate control.

The protein content of mitochondrial and submitochondrial preparations was determined according to Schacterle and Pollack [17].

The polyanion was prepared by polymerizing its components following the method described by Völker [18]. 0.25 mol methacrylic acid, 0.5 mol maleic acid anhydride and 0.75 mol styrene were added to 330 ml toluene containing 1.77 g benzoylperoxide as catalyst. The mixture was kept in a water-bath of 85 °C under reflux for about 3.5 h. The suspension of the polymer formed was cooled to room temperature, filtered, washed with toluene on a G-4 glass filter and finally dried at 105 °C. The yield was about 88 %. The polymer was dissolved in water, its pH was brought to 7.2 with KOH and used in this form in the experiments. Its composition was controlled with the following methods: acid content with back titration and styrene content by measuring the absorbance at 260 nm. The methacrylic acid : maleic acid anhydride ratio in the freshly prepared polymer was calculated on the basis of its

oxygen content determined by pyrolysis according to Unterzaucher [19]. There was about 8 % more styrene found in the polyanion than calculated on the basis of the composition of the starting mixture used for polymerization. There was practically no difference between the calculated and determined ratios of methacrylic acid : maleic acid anhydride present in the polymer. The average molecular weight (M_n) of the polyanion was determined after dialysis in a Knauer electronic osmometer using Sartorius 11536 membrane and found to be 10 000.

All substrates were added in the form of either Tris or sodium salt (pH 7.2), if not otherwise indicated.

The chemicals used were the purest commercially available. ADP and ATP were the products of Reanal, Budapest. [2-^{3}H]ADP was purchased from the Radiochemical Centre, Amersham. Carboxyatractyloside was obtained from Boehringer (Mannhein, GFR).

RESULTS

In Fig. 1, Expt. 1 demonstrates the effect of the polyanion on the respiration of rat liver mitochondria, in the presence of glutamate+malate, as substrates. There is a concentration-dependent inhibition of state 3 respiration by the polyanion. The inhibition increases in time. State 4 respiration was not influenced at all (Fig. 1, Expt. 3). In the presence of 100 μM DNP, the inhibition of oxygen uptake by the polyanion was much less than in the presence of ADP. In the uncoupled state, the inhibition did not change in time and it increased only slightly with the concentration of the polyanion, the maximum inhibition being 30 % (Fig. 1, Expt. 2). The more than 80 % inhibition of oxygen uptake by the polyanion in the presence of ADP was relieved by DNP to about 30 % (Fig. 1, Expt. 4). From these findings, it can be concluded that the polyanion inhibited the oxygen uptake of mitochondria by two different mechanisms: one is manifested only in state 3 (strong inhibition), and the other is detectable also in the uncoupled state (slight inhibition).

In state 3, in the presence of either succinate or 3-hydroxybutyrate as substrates, a similar inhibition of oxygen uptake by the polyanion was seen like with glutamate+malate (Fig. 1, Expts. 5, 6 and 7). On the other hand, the oxidation of ascorbate+TMPD was less than 30 % inhibited even by a high concentration of the polyanion (166 μg/ml), close to the state 4 rate of ascorbate+TMPD oxidation (Table I). This finding was in accordance with the low respiratory control ratio obtained with this artificial electron donor system. In the presence of DNP, ascorbate +TMPD oxidation was not influenced by the polyanion (Table I). Thus, the slight inhibition of oxidation by the polyanion observed in the presence of DNP with the other substrates could be explained by its effect on the respiratory chain between cytochrome *b* and cytochrome *c*. An aspecific inhibition of transport of substrate anions and phosphate across the mitochondrial inner membrane as another possibility could be ruled out by the finding that the swelling of mitochondria in the presence of isoosmotic concentration of ammonium malate, ammonium succinate, ammonium citrate or ammonium phosphate, measured according to Chappell and Haarhoff [20] was not at all inhibited by the polyanion.

Fig. 1, Expt. 8 and Table II show that the strong inhibition of oxygen uptake by the polyanion depended also on the concentration of ADP present: by increasing the

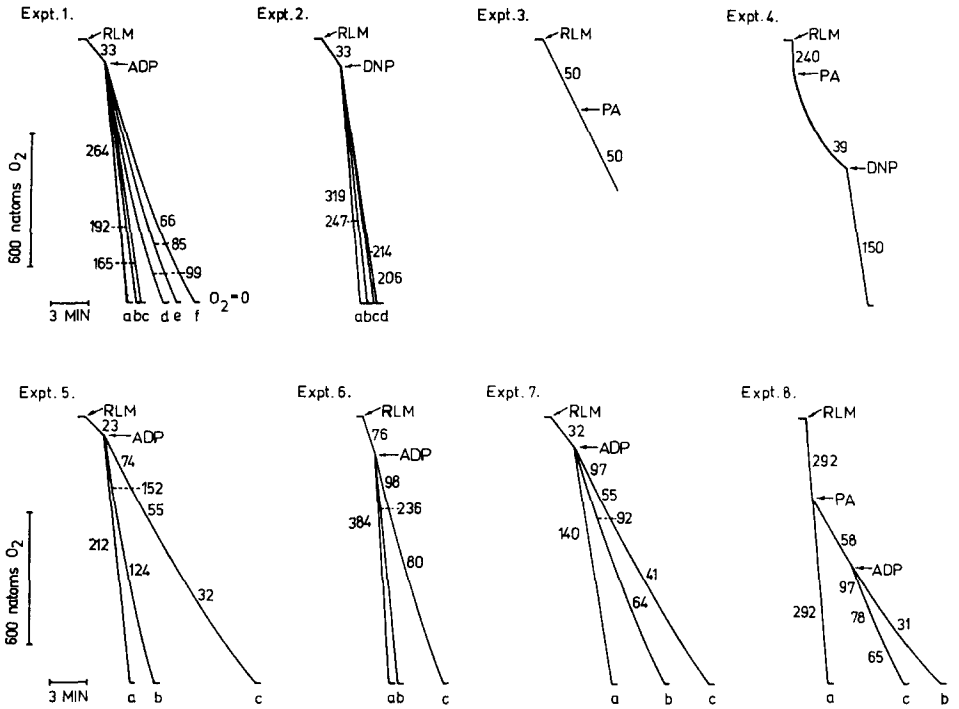

Fig. 1. Effect of the polyanion on the respiration of mitochondria under different experimental conditions. Oxygen uptake was measured polarographically in the medium described in Materials and Methods. The reaction was started by the addition of mitochondria (RLM). Expt. 1: substrates, 5 mM glutamate and 1.7 mM malate. The medium contained also: (a) 0.0, (b) 16.6,(c) 33.3, (d) 66.6, (e) 100 and (f) 167 μg polyanion (PA)/ml. 0.76 mg mitochondrial protein/ml and 2.5 mM ADP were added to the mixture at the indicated points. Expt. 2: substrates, 5 mM glutamate and 1.7 mM malate. The medium contained also: (a) 0.0, (b) 16.6, (c) 33.3 and (d) 167 μg polyanion/ml. 0.76 mg mitochondrial protein/ml and 100 μM DNP were added at the indicated points. Expt. 3: substrates, 5 mM glutamate and 1.7 mM malate. 0.52 mg mitochondrial protein/ml and 167 μg polyanion/ml were added at the indicated points. Expt. 4: substrates, 5 mM glutamate and 1.7 mM malate. The medium contained also 2.5 mM ADP. 0.75 mg mitochondrial protein/ml, 167 μg polyanion/ml and 100 μM DNP were added at the indicated points. Expt. 5: substrates, 5 mM glutamate and 1.7 mM malate. The medium contained also: (a) 0.0, (b) 33.3 and (c) 167 μg polyanion/ml. 0.88 mg mitochondrial protein/ml and 2.5 mM ADP were added at the indicated points. Expt. 6: substrate, 3 mM succinate. The medium contained also (a) 0.0, (b) 33.3 and (c) 167 μg polyanion/ml. 0.53 mg mitochondrial protein/ml and 2.5 mM ADP were added at the indicated points. Expt. 7: substrate, 10 mM 3-hydroxybutyrate. The medium contained also (a) 0.0, (b) 33.3 and (c) 167 μg polyanion/ml. 0.88 mg mitochondrial protein/ml and 2.5 mM ADP were added at the indicated points. Expt. 8: substrates, 5 mM glutamate and 1.7 mM malate. The medium contained also 2.5 mM ADP, 0.64 mgmitochondrial protein/ml, and to (b, c) 167 μg polyanion/ml and to (c) (plus) 7.5 mM ADP were added at the indicated points. Temperature, 37 °C. The numbers represent oxygen uptake expressed in natom · min^{-1} · mg^{-1} mitochondrial protein.

ADP concentration either before or after the addition of the polyanion the extent of inhibition decreased.

To get further information about the mechanism of the strong ADP concentration-dependent inhibition of the oxygen uptake by the polyanion, its effect on the

TABLE I

EFFECT OF THE POLYANION ON THE OXIDATION OF ASCORBATE+TMPD IN STATE 4, IN STATE 3 AND IN THE UNCOUPLED STATE

Oxygen uptake was measured polarographically in the medium described in Materials and Methods, containing also 6 mM ascorbate, 0.6 mM TMPD and the polyanion as indicated. The reaction was started by the addition of mitochondria (0.53 mg protein/ml). Temperature, 37 °C. The reactions were linear with time.

Added polyanion (μg/ml)	Rate of oxygen uptake (natom · min^{-1} · mg^{-1} mitochondrial protein)		
	None	ADP (2.5 mM)	DNP (100 μM)
0.0	192	312	320
33.3	192	264	314
166.6	192	216	314

TABLE II

ADP CONCENTRATION DEPENDENCE OF THE POLYANION EFFECT ON MITOCHONDRIAL RESPIRATION

Oxygen uptake was measured polarographically at 37 °C in the medium described in Materials and Methods, containing also 5 mM glutamate, 1.7 mM malate and the polyanion as indicated. The reaction was started by the addition of mitochondria (0.48 mg protein/ml). After two minutes, ADP was added in the indicated concentrations and the rate of oxygen uptake was calculated from the initial phase of state 3 respiration. (Reaction rates decreased in time as shown in Fig. 1, Expt. 1.) n.m. not measured.

Added polyanion (μg/ml)	Rate of oxygen uptake (natom · min^{-1} · mg^{-1} mitochondrial protein)	
	+ADP 0.2 mM	+ADP 0.5 mM
0.0	304	298
8.3	200	244
16.6	100	160
33.3	52	110
66.6	n.m.	68

DNP-stimulated ATPase activity of mitochondria was studied. As Fig. 2 demonstrates, the polyanion inhibited the DNP-stimulated ATPase activity of mitochondria. The extent of inhibition depended on the concentration of the polyanion with 50 % inhibition at 40 μg/ml polyanion concentration. The polyanion had no effect on the "basal ATPase activity" (i.e. measured in the absence of DNP) of mitochondria.

It is generally accepted that particles of a molecular weight of 10 000, especially if they are negatively charged, do not cross the mitochondrial inner membrane [21, 22]. Thus, it seems very improbable that in intact mitochondria the polyanion acted directly on the ATPase localized on the inner surface of the inner membrane [23]. Therefore, the inhibition of the ADP/ATP translocator seemed more likely. In fact, as Fig. 3 shows a 2-min preincubation of the polyanion with the mitochondria inhibit-

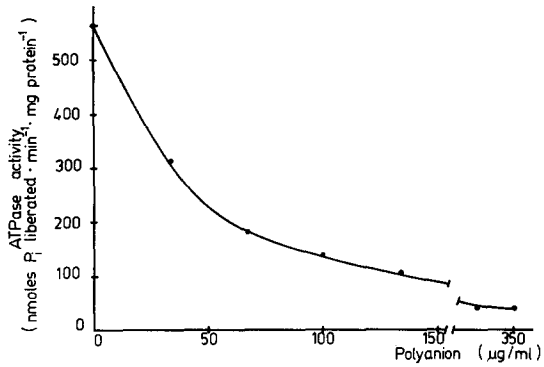

Fig. 2. Effect of the polyanion on the DNP-activated ATPase activity of mitochondria. Rat liver mitochondria (0.8 mg protein/ml) were added to the standard incubation medium. Temperature, 37 °C. After 3 min, the reaction was stopped by trichloroacetic acid and inorganic phosphate determined as described in Materials and Methods.

ed the ADP/ATP exchange. The concentration dependence of the inhibition corresponded to that of the ATPase. It is not clear why the polyanion inhibited the translocator only slightly without a preincubation period. It should be noted that at 0 °C (at which temperature the translocator activity was assayed), the interaction of the inner membrane with the polyanion might be slower than at 37 °C at which all the other reactions (including ATPase) were measured. Under the experimental conditions described, the polyanion did not increase the leakage of the labelled ADP from the mitochondria.

To check whether the polyanion had any direct effect on the ATPase, experiments were carried out with sonic submitochondrial particles, i.e. inner membrane

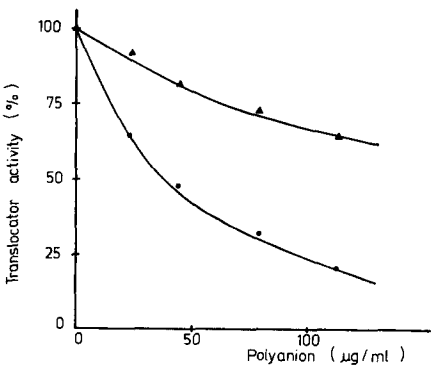

Fig. 3. Effect of the polyanion on the ADP/ATP translocator activity of mitochondria. Rat liver mitochondria were loaded with [2-^{3}H]ADP, and the translocator activity was measured by the "back exchange" method as described in the Materials and Methods. The protein concentration (mitochondria) was 3.0 mg/ml, temperature, 0 °C, the reaction time 20 s. The translocation was stopped by 25 μM carboxyatractyloside. ▲—▲, without preincubation of the mitochondria with polyanion; ●—●, after 2 min preincubation of the mitochondria with the polyanion.

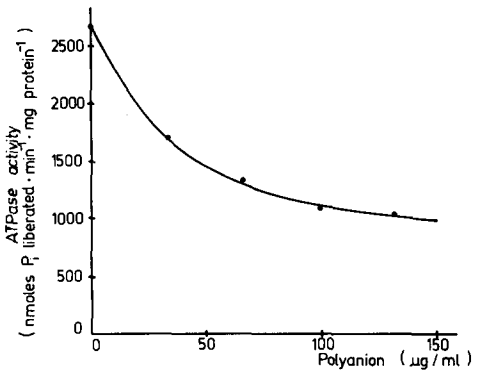

Fig. 4. Effect of the polyanion on the ATPase activity of sonic submitochondrial particles. Sonic submitochondrial particles (0.25 mg protein/ml) prepared from rat liver mitochondria as described in Materials and Methods were added to the standard incubation medium, containing also 4 mM $MgCl_2$. Other conditions were the same as in Fig. 2.

fragment vesicles turned inside-out. In these particles the ATPase was exposed to the external medium [23], therefore, it was directly accessible to both ATP and the polyanion. Since the ATPase activity of our submitochondrial preparations were completely insensitive to carboxyatractyloside, they contained exclusively inside-out vesicles. As can be concluded from the data of Fig. 4, the polyanion also inhibited the ATPase directly if their interaction was made possible. However, the inhibition was incomplete (reached approximately 60 % maximum) and it was apparent that the ATPase was somewhat less sensitive towards the polyanion than the translocator.

DISCUSSION

The results reported in this paper suggest that the copolymer of methacrylate, maleate and styrene in 1 : 2 : 3 proportion with an average molecular weight of 10 000 has at least a dual effect on intact mitochondria. It slightly inhibits the respiratory chain between cytochrome *b* and cytochrome *c* and it inhibits strongly oxidative phosphorylation and the ATPase by acting on the adenine nucleotide translocator. Besides, if an interaction is made possible, the polyanion inhibits also directly the ATPase, though incompletely and less powerfully than the translocator.

There is a profound difference between the effect of dextrane sulphate and that of the polyanion used by us on oxidative phosphorylation and ATPase. While the former acts only in sucrose or mannitol medium and has no effect in a KCl medium [6, 7], the latter acts in both media (unpublished observation). This difference might be due to the different nature of interaction of the two kinds of polyanions with the outer surface of the inner mitochondrial membrane. Dextrane sulphate interacts solely electrostatically, while in the binding of the polyanion, reported in this paper, hydrophobic forces might also be involved. This kind of interaction is also suggested for the mechanism of binding of some other inhibitors of the adenine nucleotide translocator, like the CoA-thioesters of long chain fatty acids [24–29], anionic detergents [28] and agaric acid, reported very recently [30]. The inhibitory effect of our polyanion on the adenine nucleotide translocator appears to be quite specific con-

cerning other mitochondrial anion translocators, since phosphate, dicarboxylate and tricarboxylate translocators were not affected by it. We also found that the inhibitory effect on oxidative phosphorylation and ATPase of polyanions depended significantly on their composition, especially on the styrene content of the polymer (unpublished observation). This indicates a role of hydrophobic parts of the molecule in the interaction with the membrane, and in addition, their negatively charged groups interact with positively charged groups of the adenine nucleotide translocator.

We have also checked that for the described effects of the polyanion, contaminating molecules of low polymerization grade could not be responsible. Preliminary results showed that there is practically no difference between the effects of the polyanion preparation and that of a purified fraction with the same average molecular weight obtained from the former by ammonium sulphate precipitation.

Calculations based on the release of adenylate kinase (EC 2.7.4.3) from the intermembrane space, a measure of the intactness of the outer membrane [31] indicates that, under our experimental conditions, the degree of damage to the outer membrane was less than 25 % (unpublished observations). Thus, it appears that the intact outer membrane of rat liver mitochondria is permeable to the polyanion. Therefore, the comparison of the inhibitory effect of polyanions with different structure and molecular weight on the adenine nucleotide translocator offers an approach to study both the structure of the outer surface of the mitochondrial inner membrane and that of the permeability properties of the outer membrane. This sort of experiments with intact mitochondria and mitoplasts, i.e. mitochondria without outer membrane, is in progress.

ACKNOWLEDGEMENT

Thanks are due to Mrs Éva Pocskay for her excellent technical assistance.

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