Biochimica et Biophysica Acta, 376 (1975) 458–469

BBA 46883

ATP- AND PHOSPHATE-INDUCED CONFIGURATIONAL CHANGES OF SUBMITOCHONDRIAL PARTICLES

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(Received September 9th, 1974)

SUMMARY

Light scattering was employed to monitor configurational changes of submitochondrial particles. Such changes were induced by ATP but not by analogues of this nucleotide. Mg^{2+} in an equimolar concentration to ATP enhanced the effect of the nucleotide. The ATP-induced changes were inhibited by oligomycin and uncouplers. Atractyloside was effective as an inhibitor only when loaded within the particles. The ATP-induced changes were decreased by phosphate. The effect of phosphate was partially inhibited by mersalyl. Sodium phosphate and ammonium phosphate were more effective than potassium phosphate.

The observed changes in light scattering were due to (a) events involved in energization and de-energization of the membrane, and (b) events concerning transport over the particulate membrane.

The changes were specific for adenine nucleotides and phosphate.

INTRODUCTION

Mitochondrial inner membranes undergo structural changes as a result of energization, whether the energy originates from ATP hydrolysis or from respiration in a coupled system [1–3]. Submitochondrial particles, obtained by sonic treatment of mitochondria and consisting of spherical fragments of the inner mitochondrial membrane, are exposed to the same type of energy-dependent structural changes as demonstrated by Harris et al. [4] with the light-scattering technique. Using this technique and so called "structural probes" Wrigglesworth and Packer [5] have described pH-dependent conformational and configurational changes in submitochondrial particles, and Hatase and Oda [6] have demonstrated that energization of submitochondrial systems induced disappearance of the head-piece-stalk sector from the surface of the vesicles. Papa et al. [7] have demonstrated swelling of EDTA submitochondrial particles caused by energy-linked influx of salts. Stoner and Sirak [8] have reported that the inner membrane of bovine heart mitochondria undergoes contraction when exposed to exogenous ADP, ATP or certain other high-energy phosphate

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

compounds. Some analogues of ATP were less effective than ATP in producing contraction in energized mitochondria, whereas they were equally as effective as ATP in de-energized mitochondria [8]. However, Weber and Blair [9] stated that the ADP- and ATP-induced shrinkage of mitochondria was independent of energy transfer to and from these nucleotides and that these structural changes were not induced by other purine and pyrimidine nucleotides. This specificity is in agreement with the results from other groups [8, 10]. We have demonstrated earlier [11] that submitochondrial particles obtained by sonication of beef heart mitochondria had the ability to utilize IDP and certain other nucleoside diphosphates as phosphate acceptor in addition to ADP and thus have no absolute phosphate acceptor specificity in contrast to intact mitochondria. This lack of specificity was revealed upon inversion of the inner mitochondrial membrane during sonic treatment [11–15]. It has been reported that the submitochondrial membrane alters its conformation in response to variations in energy state [6, 7] and we were thus interested to see if such changes could be accomplished by nucleoside triphosphates with a specificity similar to that observed for the energy-dependent NAD^+ reduction [16]. In the present investigation we have employed the light-scattering technique to follow the configurational changes upon energization and de-energization. We have also studied the effect of uncouplers, oligomycin and the translocase inhibitor atractyloside [17] on the membrane energization by exogenous ATP. In contrast to Harris et al. [4] we found that light-scattering changes could take place in the absence of added P_i but on the other hand that P_i could reverse the change previously induced by ATP. From the present report it is likely that energization and configurational changes of the submitochondrial membrane are due to accessibility of both the inside and the outside of this membrane to ATP, and that the reversal induced by phosphate might be explained in terms of de-energization. This reversal may involve transport of phosphate over the membrane together with a cation.

MATERIALS AND METHODS

All chemicals were of reagent grade. Nucleotides and oligomycin were purchased from Sigma Chemical Co (St. Louis, Mo., U.S.A.). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was a gift from Dr P. G. Heytler, Du Pont de Nemours, Wilmington, Del., U.S.A.

The preparation of beef heart mitochondria and the sonic irradiation of these to yield submitochondrial particles has been described earlier [18, 19]. Protein content was determined according to Gornall et al. [20]. Light-scattering changes were followed in an Eppendorf photometer with an attachment for fluorescence measurements, where the light scattered was followed at an angle of 150°. The primary filter was one of 546 nm and the secondary 500–3000 nm. Thermostability and stirring in the cuvette, volume 3 ml, were maintained automatically according to Näslund et al. [21]. The temperature was 30 °C and the basic medium contained 0.25 M sucrose and 10 mM Tris · HCl, pH 7.5.

RESULTS

It has been demonstrated by electron microscopy [6] that submitochondrial particles of beef heart mitochondria change their conformation upon energization

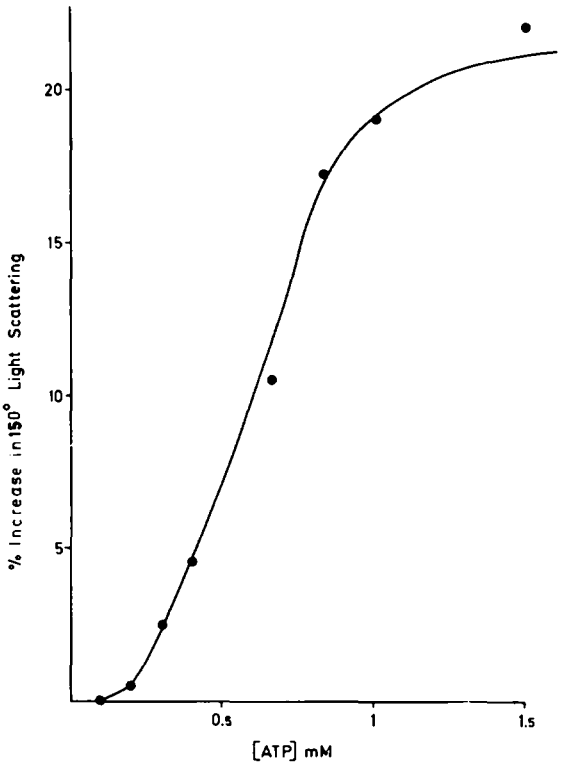

Fig. 1. Effect of ATP on light scattering of submitochondrial particles. The cuvette contained in a final volume of 3.0 ml, 0.25 M sucrose, 10 mM Tris · HCl, pH 7.5, and 0.1 mg of submitochondrial particle protein per ml and ATP as indicated. The changes in light scattering are expressed as a percentage, where 100 % reflect the increase in light scattering upon addition of the particles to the medium.

by ATP. With the light-scattering technique a corresponding change could be achieved in the absence of phosphate in contrast to the findings of Harris et al. [4] but in agreement with Papa et al. [7] in their study of energy-linked swelling of EDTA submitochondrial particles. Fig. 1 demonstrates the increase in light scattering as a function of ATP concentration in the absence of added phosphate. The maximal effect was achieved when the final ATP concentration reached 1.5 mM and concentrations of ATP tested up to 5 mM gave no further increase in light scattering. The minimal concentration of ATP which gave a measurable effect was 0.2 mM. As seen in Fig. 2, the higher the concentration of ATP the more rapid was the increase in light scattering.

Table I demonstrates the effect of various nucleotides compared to the effect of ATP. Of the nucleotides tested, only ADP could mimic the effect of ATP to a certain extent, whereas ITP and GTP seemed to have no effect. Higher concentrations of ADP gave a somewhat larger increase in light scattering. Since the adenylate kinase activity is supposed to be relatively high in this type of submitochondrial particles [22], the influence of that enzyme can not be excluded. Concentrations of ITP, up to 5 mM, did not change the amount of light scattered. This finding was un-

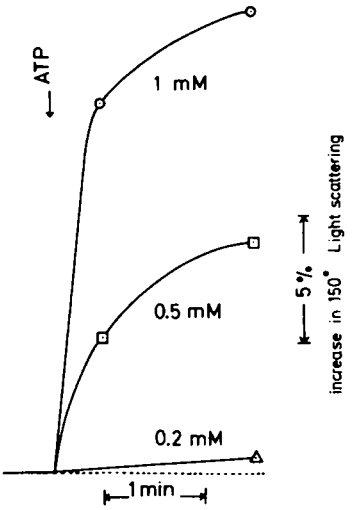

Fig. 2. Time course of increase in light scattering upon addition of ATP. Experimental conditions as in Fig. 1.

expected since ITP could substitute for ATP as the energy source for succinate-linked NAD^+ reduction [16] although with a lower degree of efficiency.

It has been demonstrated earlier that Mg^{2+} plays an important rôle in the ATP-driven NAD^+ reduction [18, 23] as well as for the phosphate acceptor specificity during oxidative phosphorylation [11]. Wrigglesworth and Packer [5] have reported that $MgCl_2$ per se at a concentration of 0.7 mM increased the light scattering of sub-mitochondrial particles by about 20 %. In their case the particles were suspended in a salt-free medium. $MgCl_2$ gave no increase when added alone to our slightly buffered system. As seen in Fig. 3 the change in light scattering induced by ATP was, however, further increased by $MgCl_2$ and a maximal effect was achieved at a concentration of 1 mM $MgCl_2$. The effect of magnesium was additionally dependent on the concentration of ATP. The best results were obtained when equimolar concentrations of Mg^{2+} and ATP were used. At higher concentrations of Mg^{2+} the effect of this ion

TABLE I

EFFECT OF DIFFERENT NUCLEOTIDES ON LIGHT SCATTERING

Experimental conditions as in Fig. 1.

Nucleotide	Concn (mM)	Changes in 150° light scattering	% of ATP-induced increase in light scattering
ATP	1	19.0	100
ADP	1	2.75	14.5
ADP	3	5.50	29.0
AMP	1	0.25	1.3
ITP	1	0.0	0
GTP	1	0.0	0

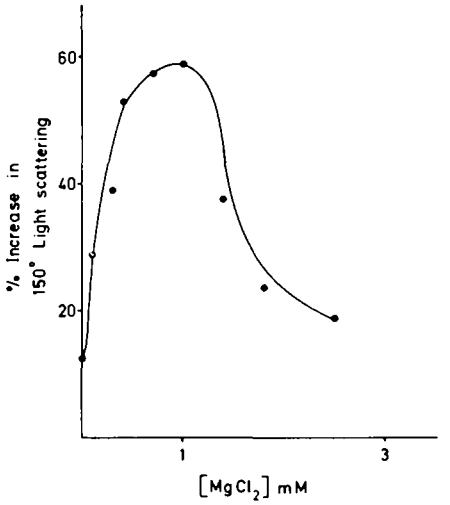

Fig. 3. Effect of $MgCl_2$ on the ATP-induced increase in light scattering. Experimental conditions as in Fig. 1, but the concentration of ATP was 1 mM and of $MgCl_2$ as indicated.

was diminished to the level where no magnesium was present. The stimulatory effect of Mg^{2+} was overcome by EDTA, when the amount of this chelating agent exceeded that of magnesium. Schuster and Olson [24] have demonstrated that $Mg^{2+} \cdot$ ATP submitochondrial particles released a significant amount of magnesium upon energization of the membranes by added ATP. This release of magnesium from the particles was either prevented or completely reversed if uncouplers were further included in the incubation medium.

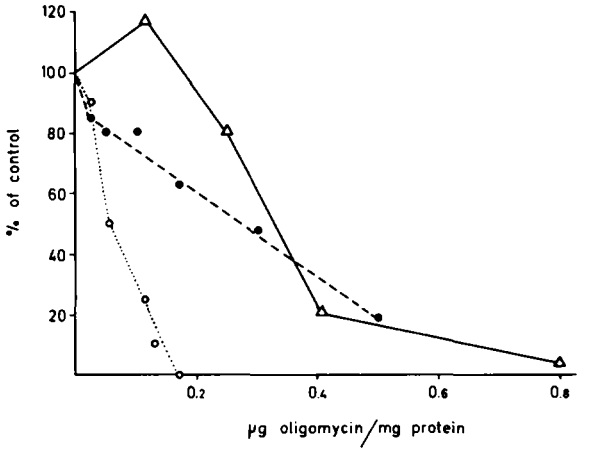

Fig. 4. Effect of oligomycin on three different systems. ●, percent increase in light scattering of submitochondrial particles. Experimental conditions as in Fig. 3. The concentration of $MgCl_2$ was 1 mM. ○, ATP-driven succinate-linked NAD^+ reduction. For experimental conditions see ref. 23. △, phosphorylative capacity during oxidative phosphorylation. For experimental conditions see ref. 11.

When the concentration of oligomycin exceeds 0.2 μg/mg protein it is known to act as an inhibitor of the phosphorylative capacity in submitochondrial particles obtained by sonication [11]. Lower concentrations of oligomycin cause a complete inhibition of the ATP-dependent NAD^+ reduction [23]. These effects have also been demonstrated with EDTA particles [25]. Fig. 4 shows a comparison of the oligomycin effect on phosphorylative capacity, ATP-driven succinate-linked NAD^+ reduction and light scattering of beef heart submitochondrial particles. This comparison clearly demonstrates that concentrations inhibiting the light-scattering system fall within the same range as those affecting oxidative phosphorylation. The same conclusions can be drawn about the effect of uncouplers. A concentration of $2 \cdot 10^{-6}$ M CCCP which fully abolished the phosphorylative capacity [19] also prevented the increase in light scattering induced by ATP.

Atractyloside on the other hand had no significant effect on the ATP induced configurational changes when added to the medium at a concentration of 50 μM. This is in agreement with Löw et al. [16] who established that energization of submitochondrial particles by ATP was largely insensitive to atractyloside. Weidemann et al. [26] had the same experience from their studies on adenine nucleotide binding. They also noticed that sonication of mitochondria in the presence of atractyloside, in order to include atractyloside within the particles, did not significantly decrease the adenine nucleotide binding. Our attempt to incorporate atractyloside within the particles resulted in the findings presented in Table II, where atractyloside-treated particles (A particles) are compared with ordinary particles (C particles). The effect of ATP on A particles was only about 35 % of that on the C particles. From Table II it is also clear that externally added atractyloside had no additional effect.

The change in pH of the particulate medium upon addition of 1 mM ATP, which gave an increase in light scattering, lay within the same range as that of Thayer and Hinkle [27] as checked with a pH electrode parallel to the light scattering. Our changes of about 0.05 pH units were far too low to account for those pH-dependent light-scattering changes of submitochondrial particles observed by Wrigglesworth and Packer [5] who found an increase in light scattering of about 10 % when the pH value was reduced from 7.5 to 7.0.

Harris et al. [4] found that P_i alone did not induce light-scattering change. Our experiments gave the same results, but phosphate counteracted the increase in light scattering induced by ATP, whether the phosphate was added before or after the nucleotide. The effect of different concentrations of phosphate is shown in Table

TABLE II

EFFECT OF ATRACTYLATE ON ATP-INDUCED INCREASE IN LIGHT SCATTERING

Experimental conditions as in Fig. 4. C particles, control submitochondrial particles. A particles, submitochondrial particles prepared with $5 \cdot 10^{-5}$ M atractyloside present in the sonication medium.

Type of particle	% changes in 150° light scattering	
	−Atractylate	+Atractylate ($5 \cdot 10^{-5}$ M)
C	51.5	50.0
A	18.8	17.8

TABLE III

EFFECT OF DIFFERENT PHOSPHATES ON ATP-INDUCED INCREASE IN LIGHT SCATTERING

Experimental conditions as in Fig. 4. Phosphate media were adjusted to pH 7.5.

Phosphate (mM)	% decrease of ATP-induced increase		
	NaP_i	KP_i	NH_4P_i
1	63	30	63
2	80	49	87
3	85	61	–
5	–	–	110
6	108	90	–

III. In these cases phosphate was added 2 min after ATP and the P_i reversal of the ATP induced increase in light scattering is tabulated. Even with higher concentrations of phosphate than those shown in this table, the maximal decrease was about 110 %. It is also obvious from Table III that there was a difference in effect between the potassium salt and the other phosphate salts employed.

In Fig. 5 a comparison of some different sodium salts is depicted. Of the anions tested only arsenate was able to mimic the effect of phosphate to any extent. Arsenate is the anion which from chemical and biochemical aspects is most similar to phosphate, thus arsenate and phosphate are competitors for a site in the respiratory chain phosphorylation mechanism [28]. Acetate, which in rat liver mitochondria penetrates the inner membrane and causes swelling, [29] had no effect, nor had chloride, when replacing phosphate, on light-scattering changes in submitochondrial particles. To investigate if phosphate in our experiments was transported over the particulate membrane, we employed mersalyl, which is known to interfere with the translocation of phosphate [30–32]. An addition of 0.1 mM mersalyl to the reaction medium diminished the effect of 1 mM sodium phosphate to about 1/3, indicating that phosphate

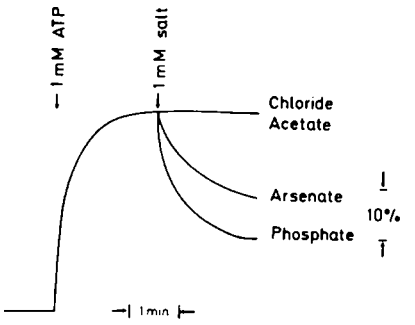

Fig. 5. Effect of some anions on ATP-induced increase in light scattering. Experimental conditions as in Fig. 4. All salts used were sodium salts with a pH value of 7.5.

TABLE IV

EFFECT OF VALINOMYCIN AND NIGERICIN ON CHANGES IN LIGHT SCATTERING

Experimental conditions as in Fig. 4. The concentration of potassium phosphate was 1 mM, of valinomycin 10^{-6} M and of nigericin $1.6 \cdot 10^{-4}$ mM.

Additions	150° light scattering		% decrease of ATP-induced increase
	$-KP_i$	$+KP_i$	
None	40.0	33.2	17
Valinomycin	46.5	34.4	26
Nigericin	42.0	32.7	22
Valinomycin + nigericin	50.0	26.2	48

has to be translocated across the membrane to be fully effective. As the membrane shows a high permeability to ammonium (as NH_3) [29], and a cation/proton exchange activity which is more active with sodium than with potassium [33], the observed cation specificity (Table III) suggests that cation entry occurs in parallel with that of phosphate. To facilitate the transport of K^+ valinomycin was added to the suspension. This ionophore is known to increase the rate of penetration across the mitochondrial inner membrane of K^+, Rb^+ and Cs^+ [29, 34]. From the results presented in Table IV it was obvious that an addition of 10^{-6} M valinomycin to the reaction medium did not significantly increase the effect of potassium phosphate. More pronounced results were achieved in a system where valinomycin as well as nigericin were present, but in this case the combination of these antibiotics in the presence of potassium act as an uncoupler [35].

DISCUSSION

Light-scattering changes follow energization of submitochondrial systems. In the hands of Harris et al. [4] such changes could be demonstrated either by addition of ATP or by substrate provided inorganic phosphate was present in the medium. The substrate induced changes required a further non-specific and rutamycin-insensitive pretreatment of the membraneous fraction by nucleotides. Under our experimental conditions phosphate counteracted the ATP-induced increase in light scattering and, in contrast to other energy-linked reactions [16], ATP was the only nucleotide effective. The discrepancy, as far as P_i is concerned, between our results and those of Harris et al. [4] might be due to differences in the experimental conditions. There is, however, no need for phosphate in ATP-driven proton translocation. In a system similar to ours, Thayer and Hinkle [27] have demonstrated that concentrations of ATP, similar to ours, gave an inward directed proton translocation.

The fact that the light-scattering increase induced by ATP was specific to adenine nucleotides may indicate that, besides the ATP hydrolysis, the adenine nucleotide translocation carrier was involved in the observed configurational changes since this carrier is highly specific for adenine nucleotides [26]. Structural changes of mitochondria induced by ADP have been demonstrated to be caused by the binding of ADP to the carrier [36]. It might thus be possible that the changes observed by the light-scattering technique upon addition of ATP may have at least two reasons: binding to the adenine nucleotide carrier and energization by ATP-driven proton

translocation.

As oligomycin (Fig. 4) as well as uncouplers inhibit the ATP-induced light-scattering changes of submitochondrial particles, this speaks in favour of energy dependent configurational changes of the particulate membrane, since binding of ATP to the adenine nucleotide carrier was not affected by uncouplers [26].

Submitochondrial particle membranes have been shown to release significant amounts of magnesium upon energization of the membranes by added ATP [24]. If such a release is a prerequisite for the energization, high external concentrations of Mg^{2+} (1.5 mM and above) may counteract the release and thus cause a decrease in ATP induced change in light scattering as observed in Fig. 3. Since the highest increase in light scattering was achieved with equimolar concentrations of Mg^{2+} and ATP, it is the ATP · Mg^{2+} complex rather than free ATP which induces the configurational changes. It should be noted in this context that free ATP rather than the magnesium complex acts as the substrate for the adenine nucleotide carrier [37].

If the energization of the particles by ATP was the only reason for the changes in light scattering, one would expect no effect of externally added atractyloside since submitochondrial particles obtained by sonic irradiation are inverted with respect to the mitochondrial inner membrane and therefore do not need any transport system for ATP. This is in agreement with our findings (Table II). However, sonication of mitochondria in the presence of 50 μM atractyloside, which should then be occluded within the particles formed, significantly lowered the ATP induced increase in light scattering. This effect could not be enhanced by additional externally added atractyloside (Table II). In sonic particles externally added atractyloside had no effect either on the ATP-driven proton translocation [27] or on the adenine nucleotide binding to the adenine nucleotide carrier [26]. Shertzer and Racker [38] have demonstrated that the ADP-dependent uptake of ATP in submitochondrial particles was strongly inhibited by 200 μM internal atractyloside, an amount in great excess (K_i was about 3 μM at 1 mM ATP), whereas externally added atractyloside had little effect. This would indicate that the inhibition of the ATP induced light-scattering increase in the atractyloside-loaded particles depend on inhibition of adenine nucleotide translocation. The conclusion must be that the configurational changes we see as changes in light scattering upon addition of ATP to submitochondrial particles have more than one reason. These changes are probably dependent on energization as well as on adenine nucleotide translocation. The effects of oligomycin and uncouplers support energization and the effect of internal atractyloside supports translocation. In favour of the latter may also be the lack of any configurational change upon addition of ITP, which could act as an energy source in energy-dependent NAD^+ reduction [16].

It is evident from our experiments (Table III) that inorganic phosphate abolished or diminished the ATP-induced effect on submitochondrial particles as seen with the light-scattering technique. The adenine nucleotide induced contraction of the inner mitochondrial membrane demonstrated by Stoner and Sirak [8] was reversed by inorganic phosphate presumably associated with a release of bound adenine nucleotides [39]. Furthermore, configurational changes of the P_i carrier which influenced the reactivity of the SH group in response to H^+ and P_i have been suggested [40]. The inhibitory effect of mersalyl suggests that, at least in part, phosphate had to be transferred to the inner side of the particulate membrane, but since the adenine

nucleotide carrier has been found to be sensitive under certain conditions to SH reagents[41,42] a direct effect on the nucleotide translocator could not be excluded. Such a direct effect is, however, less likely in our system since mersalyl had no significant effect on the ATP-induced increase in light scattering when added to a phosphate free medium. The fact that P_i reversed the ATP-induced light-scattering increase to the starting level but not significantly below indicates that our freshly prepared particles were normally in an expanded state. The evidence that inorganic phosphate per se caused the decrease of the ATP induced configurational changes in our system is based on the fact that only arsenate of the anions tested was capable to mimic this effect (Fig. 5). It is also evident that the effect of phosphate was coupled to transport of cations over the particulate membrane (Table III). This transport involved probably an exchange reaction with protons. Cockrell [43] has demonstrated that submitochondrial particles prepared by sonication in the presence of ammonia possessed a more active Na^+ for H^+-exchange system than $K^+ : H^+$ exchange. With $Mg^{2+} \cdot$ ATP particles the anaerobic release of protons taken up by respiration depended upon the cationic species, being greater with Na^+ than with K^+ [44]. This selectivity is enhanced in the presence of Mg^{2+} [45]. Van Dam et al. [46] have demonstrated energization of submitochondrial particles upon addition of valinomycin due to a passive influx of potassium. In addition energization of the membrane by ATP could be further enhanced by the addition of nigericin in the presence of potassium [46]. These facts are in agreement with our results presented in Table IV, where only the combination of nigericin plus valinomycin gave significant changes. This cooperative action of the antibiotics in uncoupling is K^+ dependent [47] which explains the lack of decrease of ATP-induced increase in light scattering in a potassium-free medium with the two antibiotics. It is obvious that the light-scattering changes observed upon addition of phosphate reflect more than one event. One may be caused by transport of phosphate over the membrane or by configurational changes of the phosphate carrier. This was indicated by the inhibitory effect of mersalyl as well as by the lack of effect of other ions freely transportable over the membrane. An other event is a de-energization of the membrane by the movements of cations over the membrane accompanying the phosphate transport or by the uncoupling effect in the presence of the two antibiotics nigericin and valinomycin. From our results it is not possible to conclude which one of these events is primary or if other factors, such as the phosphate potential, has any importance as far as the light-scattering changes are concerned. Nor is it possible with the light-scattering technique to really differentiate between conformational, configurational or volume changes of the particles.

To sum up the findings from our experiments it is evident that the changes of the submitochondrial membrane, as seen with the light-scattering technique, depend on the one hand on events involved in energization and de-energization of the membrane and on the other on events concerning transport over the particulate membrane. It is also evident that these changes are specific to adenine nucleotides and inorganic phosphate. The energization of the particulate membrane seems to imply other events than the changes detectable by the light-scattering technique, since nucleoside triphosphates other than ATP, e.g. ITP and GTP, can act as a source for energy-dependent reactions in submitochondrial particles, as mentioned above. This may indicate that energization of submitochondrial particles does not necessarily involve configurational changes of the particulate membrane.

ACKNOWLEDGEMENTS

The author is indebted to Dr I. Vallin for stimulating discussions. Some of the results in this paper was presented during the IUB 9th International Congress of Biochemistry in Stockholm 1973. This work was in part supported by the Swedish Natural Science Research Council.

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