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LIGHT-SCATTERING STUDIES ON RABBIT BRAIN MICROSOMES

II. EFFECTS OF ATP AND CHELATION OF Mg^{2+} ON MICROSOMAL CONTRACTION

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SUMMARY

Addition of ATP to a microsomal suspension in 0.25 M sucrose containing K^+ caused a significant decrease in light scattering from the suspension. By comparing such an ATP-induced change in the angular scattering pattern with that of osmotically induced microsomal shrinking, as well as in relation to changes in microsomal water content, the ATP-induced decrease in light scattering was proved to be due to the shrinkage of microsomal vesicles. The effect of ADP was nearly identical to that of ATP, while AMP showed practically no effect. In view of the fact that both active nucleotides are capable of chelating Ca^{2+} and Mg^{2+}, various chelating agents such as EDTA were examined and were found to cause a decrease in light scattering, the extent of which varied linearly with the logarithm of their association constant for Mg^{2+}. Such a rule was found to apply to ITP and AMP, but not to ATP and ADP. Moreover, the presence of Na^+, K^+, Li^+ or Rb^+ in the suspension was required for the occurrence of such a specific effect of ATP and ADP. Thus it was concluded that factors other than their action in chelating Mg^{2+} contribute to the contraction of microsomal vesicles induced by ATP and ADP. The action of these nucleotides was not affected by ouabain but was depressed by *p*-chloromercuribenzoate, indicating that some enzymic mechanism might be involved.

INTRODUCTION

It has been suggested that the microsomal fractions from brain and liver react as osmometers to alterations in the tonicity of the suspending medium[1–4]. In our previous report we presented a method of quantitatively estimating volume changes of microsomal vesicles by angular light-scattering studies, and it was demonstrated with this procedure that the Boyle–van 't Hoff relation applies to the osmotic behavior of brain microsomes. Such osmotic volume changes of microsomes accompanying turbidity changes appear to correspond to low-amplitude turbidity changes observed in mitochondrial suspensions[6].

On the other hand, it has been well established by light-scattering measurements, that there exists a close correlation between mitochondrial swelling and con-

traction and the process of oxidative phosphorylation: this is manifested in the so-called high-amplitude turbidity changes. Even in the mitochondrial membrane fragments, PACKER AND TAPPEL[7] demonstrated light-scattering changes due to structural alterations. Taking these facts into consideration, PACKER AND RAHMAN[8] performed light-scattering studies of reversible structural changes induced by ATP and certain detergents in a liver microsomal system. They reported that ATP and detergents led to a decrease in light scattering in the particle preparations and elevated the activity of certain enzymes, but the nature of such changes was not explored. Later AGHAJANIAN[9] reported that incubation with ascorbate *plus* AMP caused a turbidity change in the suspension of brain microsomes which accompanied a structural change in the membrane fragments. Turbidity changes occurring on addition of ATP or EDTA were also studied by ROBINSON with microsomes of rat liver[3] and brain[10] in a series of turbidimetric investigations in which the effects of these agents were considered in terms of cation chelation rather than enzymic alteration.

Such circumstances prompted us to reexamine the effects of ATP and some chelating agents on brain microsomes by angular light-scattering measurements. The experiments reported here are chiefly concerned with microsomal shrinking induced by ATP especially as compared with that caused by chelating agents.

METHODS

Essentially the same methods as those employed in our previous work[5] were applied in this study.

Microsomal suspensions were usually prepared from rabbit brain homogenized in 0.25 M sucrose as described. In some experiments, however, brains were homogenized in 10 vol. of 0.125 M KCl solution containing 20 mM Tris–HCl buffer (pH 7.3); the salt solution was only used for homogenization and washing, the final microsomal pellets being suspended in buffered 0.25 M sucrose and stored for use in the cold.

Angular-scattering measurements, protein assay and determination of the water content of microsomes were carried out as described previously.

The magnesium content of microsomal pellets was determined with an atomic absorption spectrometer (Hitachi 207), with the wavelength control initially set at 285 mμ then adjusted to obtain maximum transmission at the resonance line of magnesium 285.2 mμ.

The nucleotides used (ATP, ADP, AMP and ITP) were obtained from Sigma Chemical Co.. The chelating agents EDTA, glycoletherdiaminetetraacetate, 1,2-cyclohexanediaminetetraacetate and nitrilotriacetate were obtained from Dojin Pharm. Lab., Japan. After these reagents were dissolved in distilled water, the resulting solutions were adjusted to pH 7.3 with 100 mM Tris, submitted to centrifugation and then passed through a Millipore filter under positive pressure before use.

RESULTS

Effects of adenine nucleotides on light scattering

When ATP was added to microsomes prepared in 0.125 M KCl solution and suspended in 0.25 M sucrose, the intensity of light scattered at 45° (I_{45}) or 90° (I_{90}) showed a rapid decline, attaining a steady level within 2–3 min. On the addition of

ADP, a very similar effect was observed, while AMP showed practically no significant effect (Fig. 1).

Such a decrease in I_{45} (ΔI_{45}) induced by the addition of adenine nucleotides is dependent on the amount added, reaching a maximum level at a final concentration of about 0.5 mM ATP for 100 μg microsomal protein per ml (Fig. 2). In Fig. 2 osmotically induced ΔI_{45} (measured by suspending aliquots of the same microsomal preparation in various sucrose concentrations) is also illustrated; this indicated that the maximum ΔI_{45} induced by ATP is equivalent to that osmotically induced by 0.85–0.9 M sucrose.

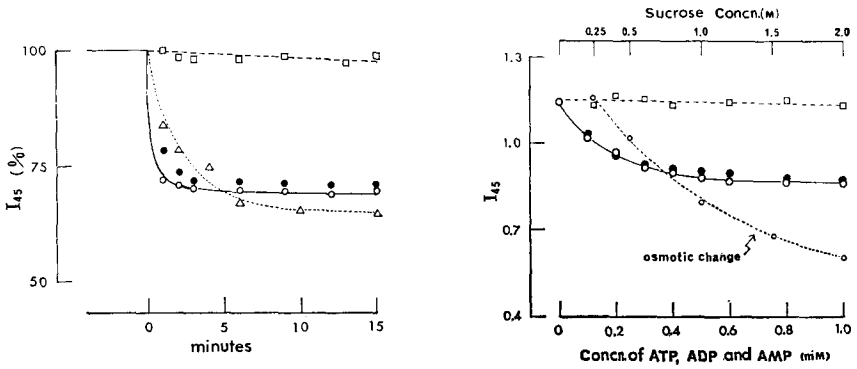

Fig. 1. Effect of adenine nucleotides and EDTA on light scattering from rabbit brain microsomal suspension. Ordinate, I_{45} at 436 mμ expressed as % of that of the control suspension; abscissa, time (min) which elapsed after adding reagents. The final concentration of added ATP (○), ADP (●), AMP (□) and EDTA (△) was 0.5 mM. Microsomes were prepared in 0.125 M KCl and suspended in 0.25 M sucrose (104 μg protein per ml).

Fig. 2. Dependence of I_{45} at 436 mμ of brain microsomal suspension on the concentration of added ATP (○), ADP (●) and AMP (□). o – – – o, osmotic volume changes observed on aliquots of the same microsome preparation suspended in hypertonic sucrose media, the concentrations of which are indicated above. Microsomes were prepared in 0.125 M KCl and suspended in 0.25 M sucrose (117 μg protein per ml).

ATP-induced scattering changes and shrinkage of vesicles

When ATP was added to microsomes prepared in 0.25 M sucrose and suspended in a sucrose medium, the change in I_{45} was not so remarkable. As seen in Fig. 3, however, an immediate decrease in I_{45} occurred when a small additional amount of K^+ (at a final concentration of 5 mM or more as KCl) was added. Alternatively, addition of KCl (5 mM) to the microsomal suspension did not cause any significant changes in I_{45}, but a further addition of ATP induced a significant decrease in I_{45}. It is obvious, therefore, that K^+ is needed to obtain maximal ATP-induced microsomal shrinkage.

K^+ could be replaced by other monovalent ions, *e.g.* Na^+, Li^+ and Rb^+, with Na^+ and K^+ being nearly equivalent for obtaining ATP-induced contraction (Fig. 3).

Because of the importance of Na^+ and K^+ for the activity of (Na^+-K^+)-dependent ATPase, reportedly present in brain microsomes[11], the effects on scattering of adding combinations of these ions in various proportions were examined. However, we failed to obtain any significant increase in ATP-induced scattering changes. Moreover, microsomes incubated with ouabain ($3 \cdot 10^{-4}$ M) for 30 min at 25° never lost their response to ATP. It might be concluded, therefore, that (Na^+-K^+)-dependent ATPase is not involved in this microsomal contraction.

As discussed in our previous report[5], if ATP-induced changes in the light scattering are really caused by shrinkage of microsomal vesicles, the angular light-scattering pattern of microsomes suspended in 0.88 M sucrose should be nearly identical with that in 0.25 M sucrose solution containing K^+ and enough ATP to cause the maximal ΔI_{45}. Indeed, as shown in Fig. 4, both curves were found to overlap almost completely for the angle of 45–135°. Applying the results presented in the previous report, therefore, it might be said that microsomal vesicles maximally shrink to about 70% of their original volume when treated with adenine nucleotides.

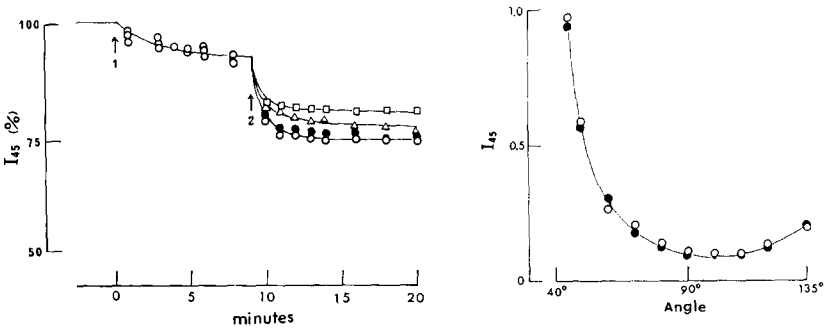

Fig. 3. Effect of monovalent ions on ATP-induced scattering changes (I_{45} at 436 mμ). At arrow 1, ATP (0.5 mM) was added. At arrow 2, Na^+(○), K^+(●), Li(△) or Rb^+(□) was further added (5 mM as chloride). Microsomes were prepared in 0.25 M sucrose and suspended in 0.25 M sucrose (126 μg protein per ml).

Fig. 4. Angular variation curves of light scattering at 436 mμ from brain microsomal suspension (108 μg protein per ml) prepared in 0.125 M KCl. ○—○, in 0.25 M sucrose solution containing 0.5 mM ATP; ●—●, in 0.88 M sucrose solution.

Alternatively, shrinkage of vesicles could be evaluated by using the observed dissymmetry coefficient (Z) as reported earlier[5]. With this method, the maximal shrinkage induced by the appropriate amount of ATP was expected to be about 25%, a value in fairly good agreement with the 30% estimated by the above method of overlapping.

Thus it was demonstrated by the light-scattering measurements that ATP caused microsomal shrinkage accompanying a decrease in turbidity. Indeed, as shown in Table I, microsomes incubated at 25° for 10 min in 0.125 M KCl medium containing ATP showed a decrease in wet weight, but their dry weight remained fairly constant, a fact indicating that ATP caused water loss from the microsomes across the membrane. Volume changes estimated from water loss are also consistent with those evaluated from the light-scattering measurements.

TABLE I

ATP-INDUCED CHANGE IN WATER CONTENT OF MICROSOMES

ATP (mM)	*Microsomal pellet (mg)*		*Water content*	
	Wet	*Dry*	*mg*	%
0	150.5±0.4	12.4±0.2	138.2±0.5	100
1.5	120.2±0.5	12.7±0.1	107.5±0.6	77.7±0.1
3.0	106.0±0.4	12.7±0.1	93.3±0.3	67.4±0.1

Divalent cations

As described previously[5], divalent cations such as Mg^{2+} and Ca^{2+} caused an increase in light scattering from microsomes suspended in sucrose media. The presence of a monovalent ion was found to exert almost no effect. The interaction of ATP and Mg^{2+} on scattering has already been reported by PACKER AND RAHMAN[8]. As stated by these investigators, the scattering decrease with ATP in the presence of K^+ was found to be reversed by Mg^{2+} or Ca^{+2} (added as the chloride) and a number of such decreases and increases could be repeated by successive additions of ATP and Mg^{2+}.

Chelating agents

ROBINSON[10] considered the effect of ATP on turbidity changes of microsomal suspensions in terms of its chelation of divalent cations. Indeed, the ability of adenine nucleotides to chelate Mg^{2+} and Ca^{2+} is well-known[12–15], and their association constant to form complexes has already been reported[13]. The above-stated interaction of ATP and Mg^{2+} might be interpreted in terms of such a chelation. To elucidate the nature of physical changes indicated by scattering, the effects of some chelating agents on light scattering from microsomal suspensions were examined. The agents used were EDTA, glycoletherdiaminetetraacetate, 1,2-cyclohexanediaminetetraacetate and nitrilotriacetate.

All these agents caused a decrease in light scattering quite similar to that induced by ATP in the presence of K^+ or Na^+ (Fig. 1). However, their action was entirely independent of whether microsomes were prepared in 0.25 M sucrose or in 0.125 M KCl as well as whether or not monovalent ions were present in suspension media.

To correlate scattering changes with chelation of divalent ions, these chelating agents and nucleotides were added to aliquots of the microsomal suspension in 0.25 M sucrose containing 95 μg per ml; their final concentrations were all adjusted to 0.5 mM, and the observed decrease in I_{45} (ΔI_{45}) was compared with their K_{Mg} and K_{Ca} values as reported by WALAAS[13].

When microsomes prepared in 0.25 M sucrose were used, ΔI_{45} was found (Fig. 5), to vary almost linearly with log K_{Mg} but no correlation was obtained with log K_{Ca}. Addition of KCl (5 mM) caused hardly any effect upon such a linear correlation of scattering changes with log K_{Mg} except for ATP and ADP; ΔI_{45} due to both nucleotides were markedly enhanced, to which the linear relation with log K_{Mg} no longer applied.

When microsomes were prepared in 0.125 M KCl solution and suspended in 0.25 M sucrose, scattering changes induced by ATP and ADP in the absence of exogenously added K^+ were also far larger than those expected by their K_{Mg}. These results strongly suggest that the scattering decrease caused by chelating agents is different from that caused by ATP in the presence of K^+, for which only a minute amount of K^+ exogenously added or remaining attached to vesicles is needed.

Treatment with these agents caused a decrease in microsomal Mg^{2+} content paralleling their K_{Mg} values. In Fig. 6 are given the results of an experiment in which microsomes prepared in 0.125 M KCl solution were incubated with these agents (1 mM per mg microsomal protein) in 0.25 M sucrose at 25° for 30 min; the pellets separated by centrifugation at 78000$\times g$ for 60 min were washed once by resuspending in 0.25 M sucrose, then spun down, and the Mg^{2+} content was determined after com-

plete dissolution (by sonication) into deionized redistilled water of known quantity. Taking into account the results of scattering studies stated above, it might be said that the scattering decreases caused by EDTA and other chelating agents including ITP parallel decreases in microsomal Mg^{2+}, but those caused by ATP and ADP in the presence of K^+ do not.

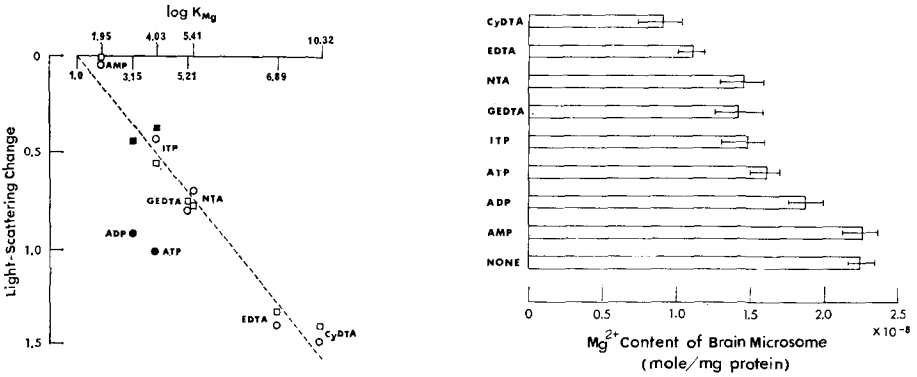

Fig. 5. Correlation between light-scattering change (ΔI_{45} at 436 mμ) of microsomal suspension (95 μg protein per ml of 0.25 M sucrose) induced by nucleotides (ATP, ADP, AMP and ITP) or chelating agents (EDTA, glycoletherdiaminetetraacetate (GEDTA), nitrilotriacetate (NTA) and 1,2-cyclohexanediaminetetraacetate (CyDTA)) and their association constant with Mg^{2+} (K_{Mg}). ○ and ●, in the presence of K^+; □ and ■, in the absence of K^+. The values on the ordinate are normalized to the value of ATP in the presence of K^+. The final concentration of nucleotides or chelating agents added was adjusted to be the same (0.5 mM).

Fig. 6. Mg^{2+} content of rabbit brain microsomes after the treatment with nucleotides or chelating agents (1 mM per mg microsomal protein). For abbreviations see legend to Fig. 5.

Thus it was demonstrated that the scattering change induced by ATP in the presence of K^+ or Na^+ was different from that induced by chelation of Mg^{2+} caused by certain chelating agents.

Effects of ATP on microsomes in hypertonic media

The addition of ATP to microsomes suspended in hypertonic sucrose solution also caused a decrease in light scattering as in the 0.25 M solution, a fact which demonstrates that vesicles partially shrunk by hypertonicity can still respond to ATP's effect of decreasing their volume. Applying the method described in our previous report[5], the scattering changes observed could be transformed into volume changes (v) by using the v–ΔI relationship obtained with aliquots of the suspension, where v is the relative volume referred to the vesicle volume in 0.25 M sucrose. An example is presented in Fig. 7A, in which v is plotted against the final concentration of ATP (c_A) added to each observation cell.

As shown in Fig. 7A, ATP-induced scattering changes, and therefore microsomal shrinkage, increased almost linearly with concentration of ATP even in hypertonic media, but reached a limit, v_d, below which an increase in c_A failed to cause further shrinkage; the more hypertonic the suspension media the smaller the value of v_d.

It has already been reported that Boyle–van 't Hoff's relation is applicable to the osmotic behavior of microsomes[2,5], which is expressed as

$$P(v_0 - b) = \text{constant} \qquad (1)$$

where P is the osmotic pressure of the suspension media, v_0 the volume of microsomal vesicles in equilibrium with P (in the absence of ATP), and b a constant usually called "osmotically dead space". The osmotic volume work done by ATP (W) might therefore be estimated by

$$W = \int_{v_0}^{v} P\mathrm{d}v = K \ln \frac{v - b}{v_0 - b} \tag{2}$$

Applying Eqn. 1 to 2 and expanding logarithmically, W can be approximated by $P(v_0 - v)$. The value of P can be estimated from the sucrose concentration, c, as in the previous report, while $v_0 - v$ is easily obtained from Fig. 7A. As shown in Fig. 7B, W is

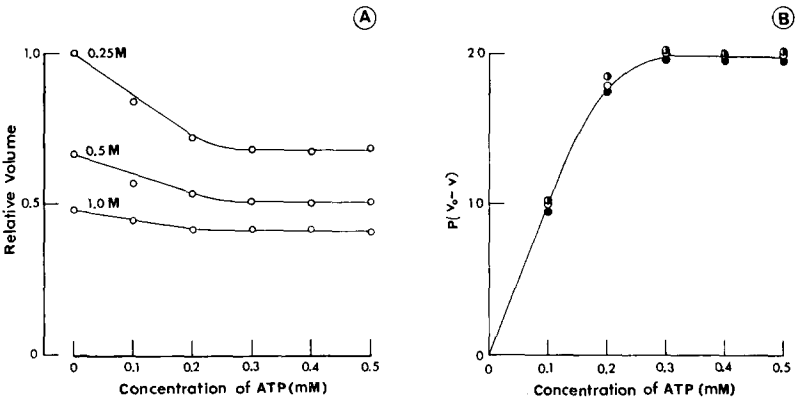

Fig. 7. (A) ATP-induced volume change of microsomal vesicles in sucrose medium of various concentrations. Ordinate, volume changes of microsomal vesicles estimated from light-scattering changes; abscissa, final concentration of added ATP. (B) Relation between the osmotic volume work done by ATP and concentration of the nucleotides derived from (A). ○, in 0.25 M sucrose; ◑, in 0.5 M sucrose; ●, in 1.0 M sucrose.

nearly independent of c. Moreover, it is clearly seen in Fig. 7B that W per mole ATP remains constant until v reaches v_d, the maximum shrinkage in a given tonicity of the medium; then it decreases as c_A increases, a fact suggesting that contraction induced by ATP increases proportionally with the number of ATP molecules per vesicle (N). However, there is a limit beyond which any further increase in N is unnecessary to elicit such a contraction.

Effects of enzyme inhibitors

As stated above, ATP-induced shrinkage could not be considered only in terms of its chelation of Mg^{2+}; some enzymic mechanism seems to be involved. The effects of some inhibitors were therefore examined: included were cyanide (10^{-4} M), azide (10^{-4} M), phloretin 2'-glucoside (10^{-4} M), α-dinitrophenol (10^{-4} M), ouabain ($3 \cdot 10^{-3}$ M) and *p*-chloromercuribenzoate (10^{-6}, 10^{-5} M).

After incubation at 37° for 30–60 min, scattering changes induced by ATP in the control suspension (0.25 M sucrose containing 5 mM KCl) were significantly depressed, so that effects of added inhibitors were difficult to ascertain. When incubated at 28–30° for 30–60 min, this problem was not encountered. However, the effect of ATP was found to be practically unaffected by the presence of the inhibitors tested, the only exception being *p*-chloromercuribenzoate. As already noted by ROBINSON[10], a

scattering decrease (about 15 %) was caused by *p*-chloromercuribenzoate alone. As shown in Fig. 8, however, percent decrease in I_{45} induced by ATP and K^+ was depressed to the level which was attained in the absence of K^+. It might be said, therefore, that the effect of ATP *plus* monovalent ions is blocked by this inhibitor, a finding which suggests that some enzymic mechanism might be involved.

On the other hand, AGHAJANIAN[9] already reported that in the presence of ascorbate AMP caused a remarkable decline in the turbidity of microsomal suspension. We have confirmed that in the presence of AMP, ADP or ATP, not only ascorbate but also cysteine, NADH and NADPH induced a decrease in I_{45} for the microsomal suspension in 0.25 M sucrose and that such scattering changes could be caused in the

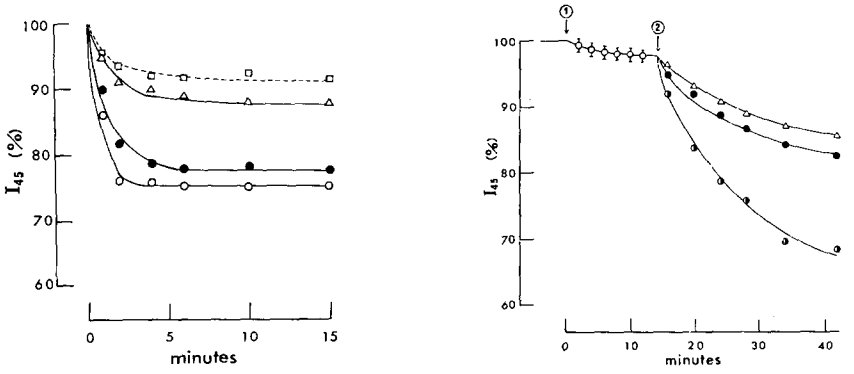

Fig. 8. Effect of *p*-chloromercuribenzoate on ATP-induced change of light scattering from microsomal suspension (121 μg protein per ml). Ordinate, I_{45} at 436 mμ expressed as % of the initial value; abscissa, time (min). □, in the absence of K^+; ○, in the presence of K^+(10 mM); ● and △, in the presence of K^+ and *p*-chloromercuribenzoate (10^{-6} and 10^{-5} M, respectively).

Fig. 9. Light-scattering changes induced by adding ascorbate and nucleotides. Ordinate, I_{45} at 436 mμ expressed as % of control value; abscissa, time (min). Ascorbate was added at first at arrow 1 (0.5 mM). Nucleotides (0.5 mM) were followed at arrow 2. △, AMP, ◑ and ○, ATP in the absence and presence of *p*-chloromercuribenzoate (10^{-5} M), respectively.

absence of K^+ or Na^+ and markedly inhibited by *p*-chloromercuribenzoate on incubation at 28° for 30 min (Fig. 9). From this time recording, however, it is obvious that the scattering decreases observed with these reducing agents are far slower than those obtained with K^+ and ATP or EDTA. It might be said, therefore, that the scattering changes caused by the former are different from those caused by the latter. A detailed report on this subject will be pulished later.

DISCUSSION

The results presented above clearly demonstrate that ATP causes decreases in light scattering from the microsomal suspension, as reported by earlier investigators[3,8,10], and that the scattering changes are really due to microsomal shrinkage accompanying water movements across vesicular membrane.

Noting that ATP and other triphosphates (but not AMP) elevated the activity of certain microsomal enzymes, PACKER AND RAHMAN[8] stated that the nature of the light-scattering changes was unknown but that such changes lead to some type of structural change in microsomes which may modify enzyme activity. ROBINSON[10], on

the other hand, suggested that the effect of ATP on turbidity changes, which could be reproduced in part by EDTA, reflected the chelation of divalent ions with a subsequent alteration in membrane permeability, rather than an enzymatic utilization of ATP.

As demonstrated above, chelating agents such as EDTA and 1,2-cyclohexanediaminetetraacetate were indeed effective in inducing scattering changes, their potency paralleling the association constant of their complex with Mg^{2+} (K_{Mg}). It should be noted, however, that the effect of ATP or ADP in the presence of a monovalent ion is far greater than that expected from its K_{Mg}. Concerning ADP, ROBINSON[17] recently reported the presence of adenylate kinase in brain microsomes, so that the effect of ADP might be explained by the formation of ATP from ADP. At any rate, it is obvious that ATP-induced shrinkage of brain microsomes in more physiological media containing Na^+ and K^+ is not solely attributable to its chelating action, some other interactions with microsomes being involved.

Two questions now arise: (1) what role is played by microsomal Mg^{2+} in maintaining the normal physical state of microsomes, that its removal leads to structural changes, and (2) how does ATP interact with microsomes in the presence of K^+ or Na^+ to cause contraction of the vesicles. The results obtained in the present study are not able to afford any satisfactory explanation of these problems, but can be commented upon.

Exogenously added Mg^{2+} has already been reported to cause scattering increases from microsomal suspension[3,5], which could reverse the decreases in scattering induced by ATP[8]. The present study confirmed these results. Such an effect of Mg^{2+} might be attributable to aggregation of the particles[17]. From our previous observations, however, it seems unlikely that microsomes aggregate to a considerable extent without exogenously added Mg^{2+}. Therefore the effects of chelating agents described above seem not to be explained by the disaggregation of vesicles due to the removal of microsomal Mg^{2+}. ROBINSON[3] referred to the effects of Mg^{2+} on ribosomal particles. Electron-microscopic observations showed that there are very few ribosomes free of or attached to the membrane in our preparations, so that such a view could not apply fully to our results. According to WALLACH *et al.*[18], the shrinkage of microsomal vesicles was considered in terms of water loss from the vesicle interior caused by a reduced Donnan effect. However, we have no evidence for or against their view. On the basis of available data we may speculate that removal of microsomal Mg^{2+} leads to some type of structural change in the particles, which causes decreases of scattering.

Concerning the second point, it seems very likely, as suggested by ROBINSON[10], that (Na^+-K^+)-dependent ATPase is not involved, because the effect of ATP is only slightly influenced by ouabain. Nevertheless we cannot reject the participation of some enzymic mechanism in the ATP-induced microsomal shrinkage; the phenomena could not be attributed to the chelation of Mg^{2+}, and the osmotic work done per mole of ATP is nearly constant until saturation occurs, while *p*-chloromercuribenzoate depressed the effect of ATP *plus* monovalent ions. It appears rather probable that some enzymic utilization of ATP is involved in the ATP-induced contraction of microsomal vesicles.

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