# The Role of ATP and ATPase in the Release of Catecholamines from the Adrenal Medulla

## II. ATP-Evoked Fall in Optical Density of Isolated Chromaffin Granules

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SUMMARY

A light-scattering technique has been employed to investigate changes in isolated bovine adrenal medullary chromaffin granules associated with release of catecholamines. The optical density (OD) at 540 m$\mu$ of chromaffin granule suspensions was proportional to the concentration of granules. ATP (0.5 mM) produced a fall in OD in the presence of 0.5 mM Mg or Mn, but not in that of Ca, Ba, or Sr. ADP produced a small fall in OD which was delayed in onset, whereas AMP was ineffective. The effect of ATP was inhibited by NEM and AMP, and was abolished by 0.3 M sucrose. In all the above conditions there was a correlation between fall in OD and release of catecholamines. The results suggest that ATP produces some change in the structure of chromaffin granules leading to the release of catecholamines and that granule ATPase may be involved in the change.

INTRODUCTION

In the previous paper (1), it was established that ATP in the presence of Mg causes the release of catecholamines, ATP, and protein from isolated chromaffin granules. The hypothesis was advanced that ATP induces conformational changes in the granule ATPase, which is known to reside in the granule membrane, and thereby initiates release of the intragranular complex of catecholamine-ATP-protein.

In the present study, an attempt has been made to utilize another approach in investigating the mode of action of ATP on isolated chromaffin granules, namely the use of a light-scattering method. While techniques which measure changes in optical density have limited value in delineating precise morphological changes in isolated subcellular particles, they do provide a very convenient and rapid means for investigating a variety of influences on these structures. The approach is especially useful in analyzing the kinetics of the *in vitro* reactions.

By the use of optical density measurements, it has been possible to demonstrate a clear correlation between changes in optical density in suspensions of chromaffin granules and the release of catecholamines as caused by ATP. While the manuscript was in preparation, a preliminary account of some of the findings of the present study appeared (2).

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## METHODS

Chromaffin granules from bovine adrenal medullae were prepared as described in the previous paper (1). Incubation of the granules was carried out in a medium of the following composition (mM): KCl, 160; NaCl, 5; TES buffer, pH 7.0, 10; EDTA, 0.05. Various modifications of this medium were made and will be described under results. Incubation was started by adding 200 $\mu$l of chromaffin granule suspension (in 0.3 M sucrose) to 2.0 ml of incubation medium in a 5.0-ml capacity cuvette. This usually produced an optical density of 0.700–0.900. Optical density (OD) measurements were carried out at room temperature (25°) in a Zeiss PMQ II spectrophotometer at 540 m$\mu$. In a few experiments, OD was also followed at 420 m$\mu$. Results have been expressed in terms of OD or as percentage of control values. Catecholamines were assayed by the trihydroxyindole method (3). Nucleotides were obtained from Sigma Chemical Co. and from Boehringer-Mannheim; TES buffer ($N$-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) from Calbiochem.; NEM ($N$-ethylmaleimide) from Mann Research Lab.; and ouabain from Nutritional Biochemical Corp.

## RESULTS

### *Relation between Optical Density and Concentration of Chromaffin Granules*

In several experiments the relationship between the OD of granule suspensions and the concentration of granules was examined. There was a linear relationship between OD at 540 m$\mu$ and granule concentration (as measured by catecholamine content) (Fig. 1). This was also true at 420 m$\mu$. Hillarp (4) reported that granule suspensions at higher concentrations in sucrose media do not follow the Beer-Lambert law. During the course of incubation of granule suspensions there was sometimes a spontaneous fall in OD which rapidly reached a steady rate of decline and did not obscure changes induced by ATP.

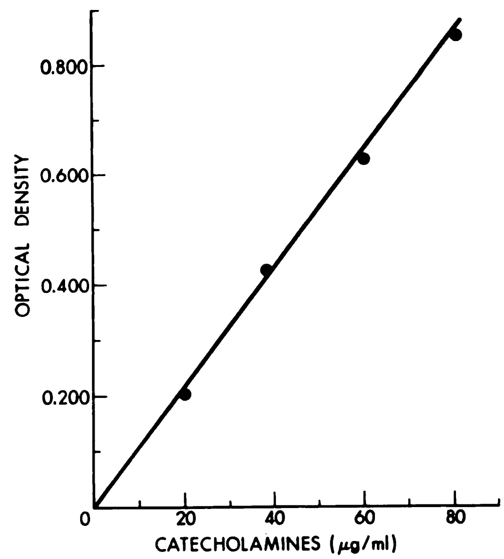


FIG. 1. *Relation between optical density and concentration of chromaffin granules*

Four different concentrations of chromaffin granules were prepared in the standard medium, which was composed of (mM): KCl, 160; NaCl, 5; TES buffer, pH 7.0, 10; and EDTA, 0.05. The ordinate represents the OD at 540 m$\mu$ and the abscissa, the concentration of granules expressed as micrograms of catecholamines per milliliter. In all the following figures, the standard medium had the composition shown above and the optical density was determined at 540 m$\mu$.

### *Effect of ATP and Mg on Optical Density of Isolated Chromaffin Granules*

The addition of Mg or ATP alone at concentrations of 0.5 mM did not produce any changes in the OD of the granules suspended in the standard medium, but the combination of these agents produced a significant fall in OD (Fig. 2). This effect of ATP in the presence of Mg was observed in 32 other preparations at 540 m$\mu$ and in each of 3 at 420 m$\mu$. A second dose of ATP did not cause any further change in the course of fall in OD (dotted arrow, Fig. 8).

### *Effect of ATP on Optical Density of Granule Suspensions of Different Concentrations*

ATP (0.5 mM) was added to granule suspensions in the presence of 0.5 mM Mg at three different concentrations of gran-

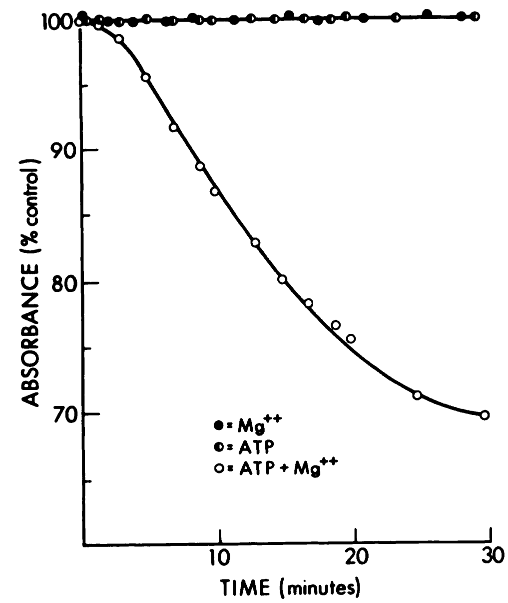


FIG. 2. *Effect of ATP + Mg on optical density of chromaffin granules*

Aliquots of a chromaffin granule preparation were suspended in the standard medium. At zero time, the following additions were made: Mg (0.5 mM), ●; ATP (0.5 mM), ◐; or ATP + Mg (0.5 mM of each), ○. The absorbance is expressed as a percentage of that found on incubation of granules with no additions.

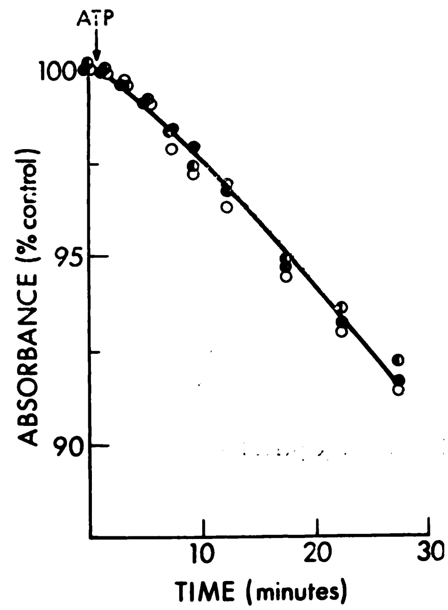


FIG. 3. *Effect of ATP on the optical density of different concentrations of chromaffin granules*

Chromaffin granules were suspended in the standard medium containing Mg (0.5 mM). Three concentrations of granules were used, corresponding to 20 (○), 40 (◐), and 80 (●) μg/ml of catecholamines. At zero time, ATP (0.5 mM) was added. Absorbance is expressed as a percentage of the initial reading before addition of ATP.

ules (equivalent to 20, 40, and 80 μg/ml catecholamines). Although the fall in OD was greater in granule suspensions with higher concentrations there was no difference in the percent fall among the three suspensions (Fig. 3).

### *Effect of Different Concentrations of ATP on Optical Density Changes of Granule Suspensions*

The OD fall produced by ATP was proportional to the concentration used when 0.125, 0.25, and 0.5 mM ATP were employed (Fig. 4). A parallel correlation between catecholamine release and concentration of ATP was reported in the previous paper (1).

### *Relation between Catecholamine Release and Optical Density Changes*

To determine whether the changes in OD of granule suspensions caused by ATP correlated with the ATP-induced release of catecholamines, as suggested above, OD measurements were made at different intervals after addition of ATP and compared to the release of catecholamines from the same preparation of granules. There was a close correlation between percent release of catecholamines and percent fall in OD (Fig. 5). For these comparisons the initial OD was corrected by subtracting the OD of the same concentration of lysed granules (see later results with hypotonicity).

### *Effect of Other Adenine Nucleotides on Optical Density of Granule Suspensions*

The addition of ADP (0.5 mM) to the granule suspension caused a small fall in OD which was variable in time of onset. In the experiment illustrated in Fig. 6 addition of ADP was followed by a 20-min delay period before any change was ob-

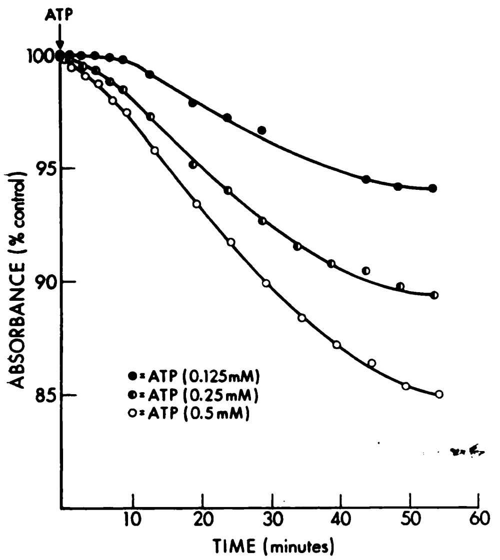


FIG. 4. *Effect of different concentrations of ATP on optical density of chromaffin granules*

Chromaffin granules were suspended in the standard medium containing Mg (0.5 mM). At zero time, ATP was added to yield three different final concentrations: 0.125, 0.25, and 0.5 mM. Absorbance is expressed as a percentage of that found with no additions.

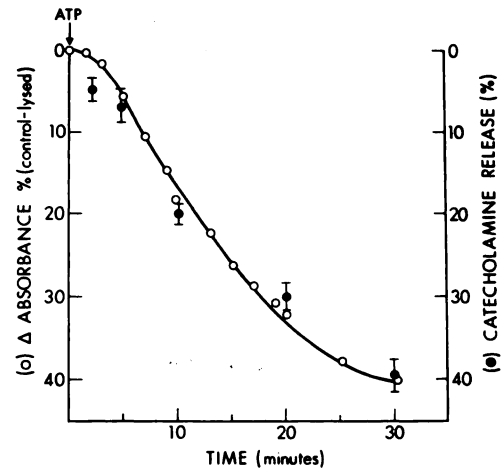


FIG. 5. *Relation between change in optical density and release of catecholamines induced by ATP*

Chromaffin granules were suspended in four tubes containing the standard incubation medium and 0.5 mM Mg. At zero time, ATP (0.5 mM) was added. At various times after the addition of ATP the absorbance was determined in one tube and the release of catecholamines in the other three. The left ordinate represents the change in absorbance (○) expressed as a percentage of the value found by subtracting from the OD of the intact granules the OD of the same concentration of granules lysed in distilled water. The right ordinate shows the percentage release of catecholamines (●) (mean ± SE).

served. Thereafter the OD fell and 60 min after addition of ADP, the fall in OD was 60% of that produced by the same concentration of ATP. In contrast, AMP produced no change at all in OD (Fig. 6).

### *Effect of Other Divalent Cations on Optical Density of Granule Suspensions*

The addition of Ca (0.5–2.0 mM) caused a small fall in the OD of the granule suspensions, but the addition of ATP (0.5 mM) did not cause any further change (Fig. 7). In contrast to this finding, Mn (0.5 mM) produced no change in OD by itself, but clearly supported the action of ATP in causing a fall in OD (Fig. 8). In other experiments it was found that Ba and Sr were unable to substitute for Mg in supporting the ATP-induced fall in OD.

### *Effect of ATPase Inhibitors on Optical Density Changes Induced by ATP*

In the previous paper (1) we suggested that the effect of ATP on catecholamine release was mediated through granule ATPase. Although ouabain, an inhibitor of plasma membrane ATPase, had no effect on ATP-induced changes in OD (Fig. 8), both NEM (Fig. 9), and AMP (Fig. 10) which block granule ATPase (1, 5) did inhibit the action of ATP on OD. The inhibition produced by NEM appeared to be noncompetitive while that of AMP was competitive in nature (Figs. 9 and 10). In higher concentrations, NEM itself produced a small fall in OD after which ATP produced no further change.

### *Effect of Hypotonicity on Optical Density of Granule Suspensions*

To confirm and extend Hillarp's findings that hypotonicity causes a fall in the OD of granule suspensions (4), the time course of OD changes in granules suspended in

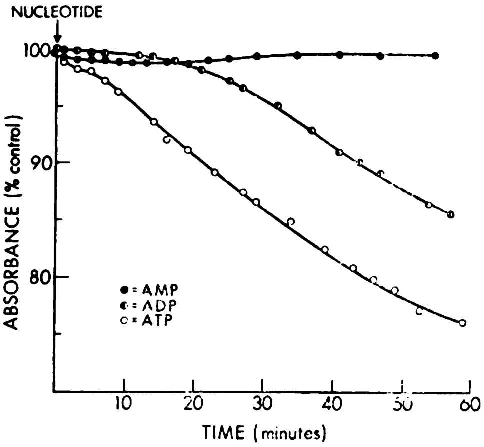


FIG. 6. *Effect of AMP, ADP, and ATP on optical density of chromaffin granules*

Chromaffin granules were suspended in the standard medium containing 0.5 mM Mg. At zero time, 0.5 mM AMP, ADP, or ATP was added. Absorbance is expressed as a percentage of that of control suspensions with no additions.

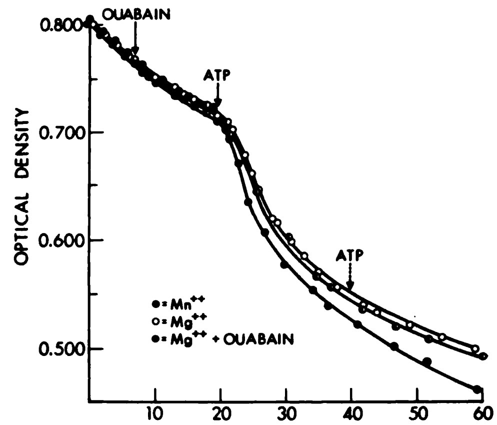


FIG. 8. *Effect of ouabain and Mn on ATP-induced optical density changes in chromaffin granules*

Chromaffin granules were suspended in the standard medium containing 0.5 mM Mg, ○ and ◐, or 0.5 mM Mn, ●. At the first arrow, ouabain ($10^{-5}$ M) was added to the preparation labeled ◐. At the second arrow, ATP (0.5 mM) was added to all three preparations; and at the third arrow, ATP was added to the preparation labeled ○. The ordinate shows the observed OD.

media of varying hypotonicity was examined. The standard medium was considered as 100% tonicity and dilutions were made with distilled water. A rapid fall in OD was observed on lowering the tonicity, approaching a maximum fall with a tonicity of 60% of the standard medium (Fig. 11). The OD of granules suspended in distilled water was about 20–30% the initial OD. After centrifugation (20,000 *g* for 20 min) of the preparation of granules lysed in distilled water, the OD of the supernatant was less than 2% of that of an equivalent amount of granules suspended in isotonic medium.

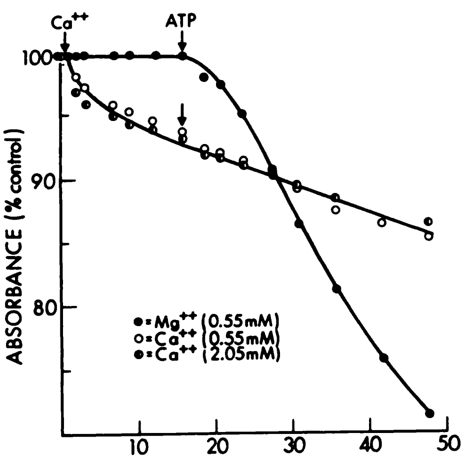


FIG. 7. *Effect of Ca, Mg, ATP, and combinations of these agents on optical density of chromaffin granules*

Chromaffin granules were suspended in the standard incubation medium. At zero time Ca (0.55 or 2.05 mM) was added to two cuvettes. The third contained Mg (0.55 mM). At 16 min, ATP was added to all three cuvettes. Absorbance is expressed as a percentage of that found with no additions.

## *Effect of ATP on Optical Density of Granules Suspended in Sucrose, Hypotonic Medium, or Water*

The addition of ATP to granules in a hypotonic medium (80%) produced a fall in OD similar to that found with regular medium. However, ATP caused no fall in OD when added to granules suspended in 0.3 M sucrose or in water (Fig. 12). In all these experiments the normal concentration of Mg was present.

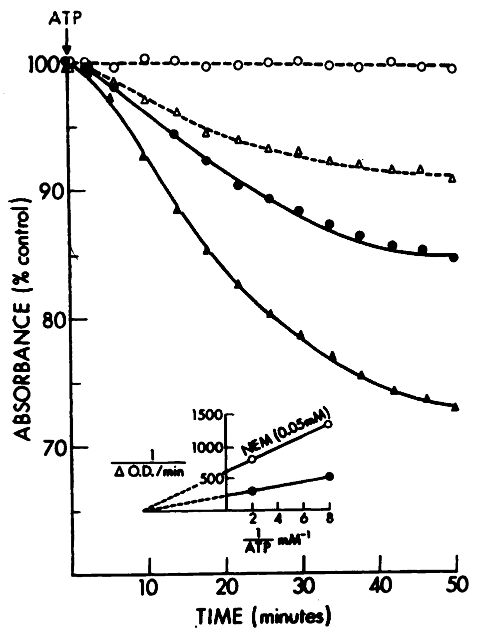


FIG. 9. *Inhibitory effect of NEM on the ATP-induced fall in optical density of chromaffin granules*

Four aliquots of a chromaffin granule preparation were suspended in the standard incubation medium containing 0.5 mM Mg. In two samples (○, △) NEM (0.05 mM) was present. At zero time, ATP was added at 0.5 mM (△, ▲) or 0.25 mM (○, ●). Absorbance is expressed as a percentage of a control sample with no additions. The inset shows the same data in a Lineweaver-Burk plot, which suggests a noncompetitive inhibition.

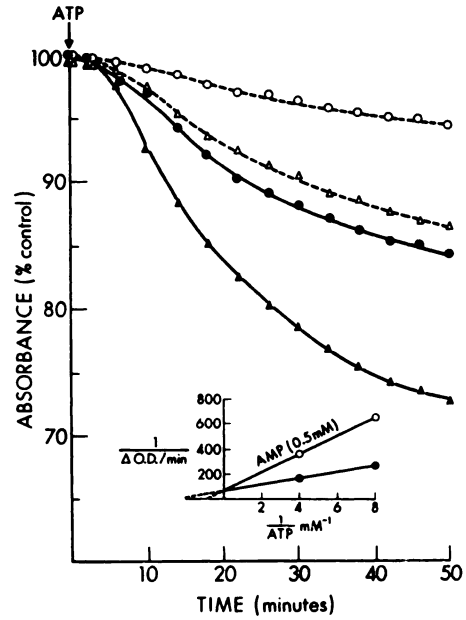


FIG. 10. *Inhibitory effect of AMP on the ATP-induced fall in optical density of chromaffin granules*

Four aliquots of a chromaffin granule preparation were suspended in the standard incubation medium containing 0.5 mM Mg. In two samples, 0.5 mM AMP (○, △) was present. At zero time, ATP was added at 0.5 mM (△, ▲) or 0.25 mM (○, ●). Absorbance is expressed as a percentage of a control sample with no additions. The inset shows the same data in a Lineweaver-Burk plot, which suggests competitive inhibition.

## DISCUSSION

Hillarp was the first to use optical density measurements to investigate the properties of chromaffin granules (4). He found that when the tonicity of the media used to suspend granules isolated in sucrose (0.3–0.9 M) was reduced there was release of catecholamines and protein and a fall in the OD of the suspension. Although the loss of amines was extremely rapid and therefore difficult to compare accurately with OD changes, he did show a similarity between the two phenomena and further showed that the changes produced by lowering the tonicity were irreversible. That the optical density at 540 mμ is due to light scattering rather than absorbance by substances in solution is shown by the fact that after lysis by hypotonicity the OD falls rapidly although the liberated substances (catecholamines, ATP, and protein) are in solution (Figs. 11 and 12). Furthermore, when the lysed granules were sedimented, the optical density of the supernatant was less than 2% of that of the original suspension in isotonic medium.

In the present study a fall in OD was produced without using such a drastic procedure as exposure to hypotonic media. Significant changes were found just by adding 0.125 mM ATP in the presence of 0.5 mM Mg. Studies on uptake of labeled catecholamines by adrenal chromaffin granules frequently have used 5 mM of Mg and ATP.

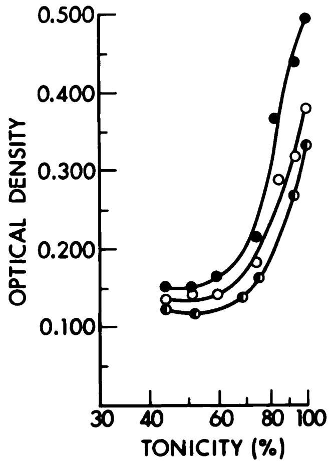


FIG. 11. *Effect of hypotonicity on optical density of chromaffin granules*

Chromaffin granules were suspended in the standard incubation medium and in dilutions of the standard medium with water. The ordinate shows the OD at 30 sec (●), and 5 (○) or 10 (◐) min after suspension of the granules. The abscissa shows the percentage of tonicity (standard medium = 100%) plotted logarithmically.

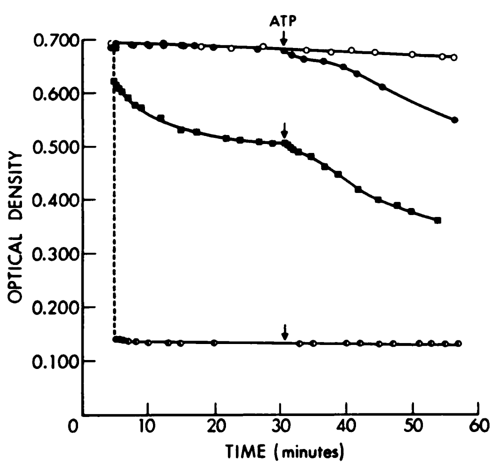


FIG. 12. *Effect of ATP on optical density of chromaffin granules suspended in sucrose, hypotonic medium, or distilled water*

Chromaffin granules were suspended in various media: standard medium (●), 0.3 M sucrose (○), standard medium diluted to 80% of normal with water (■), and water (◐). All the above media contained 0.5 mM Mg and 10 mM TES buffer, pH 7.0. ATP (0.5 mM) was added to each preparation as indicated by the arrows. The ordinate shows the observed OD.

The most striking feature of our results is that for every condition in which a fall in OD was observed (or absent) there was a direct parallel to catecholamine release. Thus ATP caused a fall in OD in the presence of Mg or Mn but not Ca, Ba, or Sr. The percent fall was proportional to the concentration of ATP but not the concentration of granules. Calcium itself (0.5 and 2.0 mM) caused a small fall with no difference between the two concentrations. AMP had no significant effect on OD while ADP had a small effect which was delayed in onset. The effect of ATP on OD was inhibited by AMP, NEM, and sucrose. NEM at high concentrations produced a small fall itself. Each of the above findings is paralleled by effects on catecholamine release (1). A more direct comparison is seen by examining the time course of the two phenomena (fall in OD *vs.* release of catecholamines). A very close correlation was observed. The delay in onset of action of ADP is consistent with production of ATP by granule adenylate kinase (6). This may explain why ADP was more effective in releasing catecholamines when incubation times were longer (1).

Optical density studies on other types of biological structures may provide clues to the action of ATP on chromaffin granules. It is known that ADP causes aggregation of platelets and this is associated with a fall in OD of platelet suspensions (7). Therefore, the fall in OD of chromaffin granule suspensions on addition of ATP might be related to the aggregation of these particles. In preliminary studies using phase contrast microscopy, no significant aggregation was observed on addition of ATP. The fall in OD after exposure to hypotonicity is also unlikely to be due to aggregation of granules. However, the action of calcium in producing a small fall in OD, which clearly differs in time course and extent from that produced by ATP, may well be related to its well-known aggregating ability. In fact calcium has been reported to cause aggregation of chromaffin granules (8).

In studies on isolated mitochondria, a fall in OD has usually been regarded as evidence of swelling, while conversely, a rise in OD has been considered to reflect contraction (9). Many agents can cause swelling (e.g., thyroxine, Ca, phosphate, etc.), but ATP has been the main substance shown to induce contraction. What can these studies tell us about swelling and/or contraction in chromaffin granule suspension? It seems unlikely that the fall in OD of chromaffin granule suspensions caused by ATP is due simply to swelling for several reasons: First, the parallelism between fall in OD and mitochondrial swelling does not always obtain (10). Secondly, the effect of ATP on mitochondria is exerted on preswollen structures where a contractile effect would be expected to lead to an increase in OD. However, there is no indication that the chromaffin granules are similarly swollen (S. Malamed, personal communication). Finally, the list of similarities between ATP-induced catecholamine release and ATP-induced mitochondrial contraction (such as metal ion and nucleotide specificity and inhibition by sucrose but not by ouabain (1, 9, 11) suggests that the ATP-induced phenomena in the two types of structures may be of the same nature, i.e., contractile. The effect of ATP on other nonmuscular structures, such as erythrocytes and amoebic vacuoles, also seems to be contractile in nature (12, 13), and indeed it has been proposed that all biological membranes possess contractility (14). This information taken together with known properties of chromaffin granules suggests a possible mechanism for the ATP-induced fall in OD.

Chromaffin granules are very dense, as revealed both by electron microscopy and by sedimentation in 1.8 M sucrose (15). The compact inner structure contributes most of the optical density since only a small fraction of the OD remains after lysis in distilled water. Therefore, when the dense inner structure (which is thought to be principally a complex of catecholamines, ATP, and protein) dissociates, the OD should also fall. In fact, the ATP-induced fall in OD coincides closely with the release of catecholamines, ATP, and protein. It is worth noting that adrenal medullary granules *in situ*, depleted of catecholamines, ATP, and protein by stimulation with acetylcholine, lose their electron density and appear to have a reduced specific gravity (16–19).

In the previous paper (1) it was suggested that ATP may produce a conformational change in the granule membrane (perhaps it is similar to the contractile effects on biological membranes cited above). The loss of granule catecholamine, ATP, and protein may be a consequence of this membrane alteration; and a fall in OD would then simply reflect the reduced structural density of the particles. Studies on possible ultrastructural changes induced by ATP are currently under investigation.

ACKNOWLEDGMENTS

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