The Adenosine-Triphosphatase Activity of Adrenal Chromaffin Granules

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1. The preparation of a fraction containing highly purified chromaffin granules from the bovine adrenal medulla is described. 2. The fraction contains an adenosine-triphosphatase activity that is stimulated by Mg^{2+} and that cannot be explained by contamination with mitochondria or microsomes. 3. It is suggested that the adenosine-triphosphatase activity is related to the uptake of catecholamines by the chromaffin granules.

Most of the catecholamines and ATP of the adrenal medulla is stored in specific cell organelles termed chromaffin granules. These granules are sedimented with the mitochondria in iso-osmotic media, but can be separated from the latter by density-gradient centrifugation. They are distinguished from the mitochondria by their lack of succinate dehydrogenase and fumarase (Blaschko, Born, D'Iorio & Eade, 1956; Blaschko, Hagen & Hagen, 1957). As the molar ratio of catecholamines to ATP in the granules is between 3 and 5, the sum of the positive charges on the catecholamines is approximately equal to the sum of the negative charges on the nucleotides. Hence it is thought that the ATP is involved in binding the amines by ionic linkages (see Hagen & Barrnett, 1960).

When the adrenal medulla is stimulated the ATP content of the gland falls as the catecholamines are secreted so that, in most instances, the molar ratio of catecholamines to ATP in the gland remains approximately constant (Carlsson, Hillarp & Hökfelt, 1957; Schümann, 1958).

Hillarp (1958*a*,*b*) reported the presence of an ATPase† activity that he claimed to be an intrinsic property of the chromaffin granules and that he suggested may be concerned in the release of the catecholamines from the granules during secretion. However, Fortier, Leduc & D'Iorio (1959) and Hagen (1962) failed to find ATPase activity in chromaffin granules.

The work described below was undertaken in an attempt to resolve the conflicting reports on the ATPase activity of the chromaffin granules. To do this it was necessary to achieve a high degree of purification of the chromaffin granules. Conditions are described that yield a fraction containing granules which was found, by electron-microscopic examination, to be devoid of significant contamination by mitochondria or microsomes.

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† Abbreviation: ATPase, adenosine triphosphatase.

METHODS

Preparation of 'large granules'. Bovine adrenal glands were removed from the carcasses within 20 min. of slaughter and placed on ice. In the laboratory the medullae were dissected from the surrounding cortical material and chopped to a fine mince with a stainless-steel knife. The mince was homogenized with 0·3 M-sucrose at 2° in a Potter–Elvehjem tissue homogenizer having a glass mortar and Perspex pestle to give a 1:5 (w/v) homogenate.

The homogenate was centrifuged at low speed (600*g*) for 20 min. in the swinging-bucket rotor for the MSE refrigerated centrifuge. The low-speed supernatant, when recentrifuged at 11000*g* (or 5000*g*) for 20 min. (see the Results section for conditions used) in the A40 rotor of the Spinco model L ultracentrifuge, gave sediments of large granules that were resuspended in 0·3 M-sucrose. These suspensions served as the starting material for the density-gradient centrifugations.

Preparation of microsomes. The high-speed supernatant, obtained by centrifugation at either 11000*g* or 5000*g*, was centrifuged at 20000*g* for 20 min. to remove any remaining large granules. The resulting supernatant was recentrifuged at 100000*g* for 1 hr. to give a sediment of microsomes. All the centrifugations were carried out in the A40 rotor of the Spinco model L ultracentrifuge.

Density-gradient centrifugation. The density gradients were prepared by layering sucrose solutions of decreasing molarity above one another 24 hr. before use. The molarities used were changed in different experiments and are described in the Results section. Immediately before centrifuging 1 ml. of a suspension of large granules in 0·3 M-sucrose was layered above each gradient. The tubes were then centrifuged at 145000*g* for 1 hr. in the Spinco swing-out rotor SW 39L. The positions of the bands of material at the end of the run were noted and the various fractions collected by using the Schuster centrifuge tube cutter.

Determination of catecholamines, ATP and protein. Catecholamines were determined by the colorimetric. method of Euler & Hamberg (1949) with the buffers used by Schümann (1957). ATP was estimated by the firefly luminescence method (see Strehler & Totter, 1954). Protein nitrogen was measured by the micro-Kjeldahl method.

Osmotic lysis of the chromaffin granules. The fraction rich in chromaffin granules was diluted with an equal volume of water and centrifuged. The resulting sediment was resuspended in 0·03M-sucrose and recentrifuged. This last step was repeated by adding fresh 0·03M-sucrose to the sediment and, after recentrifuging, the pooled supernatants were dialysed. The dialysed supernatants contained the water-soluble protein; the dialysed sediment contained the water-insoluble material.

Succinate-dehydrogenase assay. Succinate-dehydrogenase activity was estimated spectrophotometrically by measuring the reduction of cytochrome *c* at 550mμ in the presence of cyanide, as described by Kuff & Schneider (1954). As the catecholamines reduce cytochrome *c* non-enzymically it was necessary to remove all the catecholamines from the fractions by extensive dialysis against 0·03M-sucrose before carrying out the estimations.

ATPase assay. The ATPase activity of the dialysed fractions was estimated by measuring the liberation of inorganic phosphate from ATP at 37·5° in an incubation medium (final vol. 3ml.) of the following composition: tris–HCl buffer, pH7·4 (33mM); KCl (20mM); $MgCl_2$ (2·5mM); ATP (5mM). The enzymic reaction was stopped by the addition of 3ml. of 10% (w/v) trichloroacetic acid. After the precipitated protein had been removed, the inorganic phosphate liberated was measured by the method of Fiske & Subbarow (see LePage, 1957).

Electron microscopy. Fractions from the density gradients were treated with an equal volume of 2% (w/v) osmic acid solution, buffered with veronal–acetate to pH7·4, and had the same sucrose molarity as the fractions. After standing for 1hr. at 2° the fixed material was sedimented at 100000*g* for 20min. and dehydrated with ethanol. The dehydrated sediments were then prepared for electron microscopy by Dr R. Barer in the Department of Anatomy, Oxford.

RESULTS

Distribution of catecholamines, ATP and protein nitrogen between subfractions of the large granules. A density gradient was prepared by layering successive 0·5ml. solutions of 2·5M-, 2·0M-, 1·9M-, 1·8M-, 1·7M-, 1·6M-, 1·5M- and 1·4M-sucrose into a centrifuge tube and a suspension of large granules in 0·3M-sucrose was placed above it. After these tubes had been centrifuged at 145000*g* for 1hr., the opaque material was seen to be divided into two distinct bands. The upper band was yellow–brown and was at the level of the interface between the layers of 0·3M- and 1·4M-sucrose. The lower band was whitish and extended from the top of the layer of 1·6M-sucrose to the bottom of the layer of 2·0M-sucrose. The regions above and below both bands were relatively clear but the distance between the two bands was only about 0·5cm. The catecholamines, ATP and protein nitrogen of the large granules were distributed between the two bands as shown in Table 1. The protein of the large-granule fraction was about evenly distributed over the two bands, but most of the catecholamines and ATP was present in the lower band. The upper band contained 1·14μmoles of catecholamines/mg. of protein nitrogen and its molar ratio of catecholamines to ATP was 16·9. This high ratio probably indicates that most of the catecholamines in the upper band have been released from disrupted granules and that the ATP associated with them in the bound state has been degraded after release. In the lower band the molar ratio was 4·6 and there were 14·9μmoles of catecholamines/mg. of protein nitrogen. These results are in good agreement with previous observations in which it was shown that the catecholamines and ATP are recovered in a particulate fraction from the lower regions of a sucrose density gradient (Blaschko *et al.* 1957).

Table 1. *Distribution of catecholamines, ATP and protein nitrogen between subfractions derived from the large granules by density-gradient centrifugation*

Experimental details are given in the text.

Fraction	Percentage of total large-granule content in each band		
	Catecholamines	ATP	Protein N
Upper band	5·8	1·9	42·6
Lower band	76·4	91·7	43·3

Distribution of succinate-dehydrogenase activity between subfractions of the large granules. To increase the spatial separation between the upper and lower bands the density gradient was modified by inserting 1ml. of 1·55M-sucrose between the layers of 1·5M- and 1·6M-sucrose; the layers of 1·9M- and 2·0M-sucrose were omitted. In all subsequent experiments therefore the density gradients were made up as follows: 0·5ml. of 1·8M-, 0·5ml. of 1·7M-, 0·5ml. of 1·6M-, 1·0ml. of 1·55M-, 0·5ml. of 1·5M- and 0·5ml. of 1·4M-sucrose. On centrifuging suspensions of large granules over these gradients a more satisfactory separation of the two bands was achieved. The upper and lower bands of material thus obtained were tested for succinate-dehydrogenase activity. Though such activity was consistently found in the upper band it was never detected in the lower band. In four experiments the succinate-dehydrogenase activity of the upper band, in μmoles of succinate oxidized/mg. of protein nitrogen/hr., was 5·5, 3·2, 1·9 and 2·3 respectively, with a mean activity of 3·2. These experiments support previous findings that the upper band is rich in mitochondria.

Distribution of cytochrome pigments in sub-

fractions from the bovine adrenal medulla. Extensively dialysed suspensions were prepared of the upper band, of the water-insoluble material from the lower band and of the microsomes; these were dissolved in 0·1 M-glycylglycine buffer, pH 7·4, which contained sodium deoxycholate (2%, w/v). The absorption spectra of these solubilized fractions were recorded both before and after reduction with a small amount of sodium dithionite (Figs. 1 and 2). Before adding the reducing agent all three fractions showed a broad peak of absorption at about 411 mμ; this is shown in Fig. 1 for the material from the lower band (broken curve). Reduction caused the peaks at about 411 mμ in the oxidized state to shift to 426 mμ in all three fractions (Fig. 1).

The addition of dithionite caused the appearance of additional absorption peaks in all the fractions

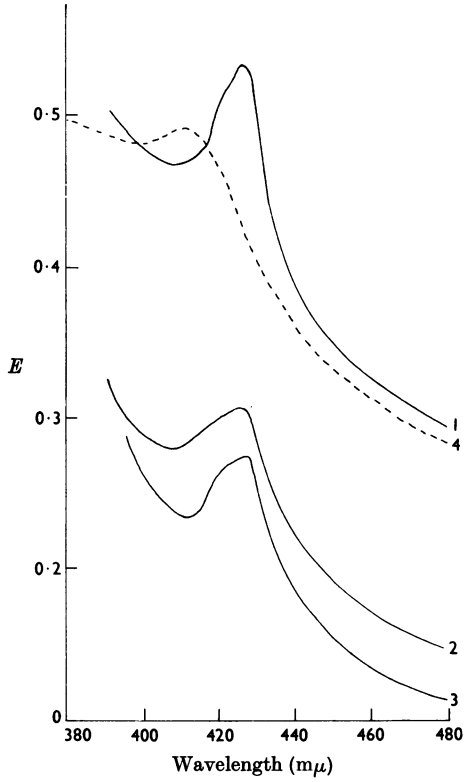


Fig. 1. Absorption spectra of cytochromes in solubilized subcellular fractions from the bovine adrenal medulla from 390 to 480 mμ measured in a Zeiss PMQIII spectrophotometer, 1 cm. path length. Curve 1: material from the lower band reduced with sodium dithionite. Curve 2: material from the upper band reduced with sodium dithionite. Curve 3: microsomal material reduced with sodium dithionite. Curve 4: material from the lower band in the oxidized state.

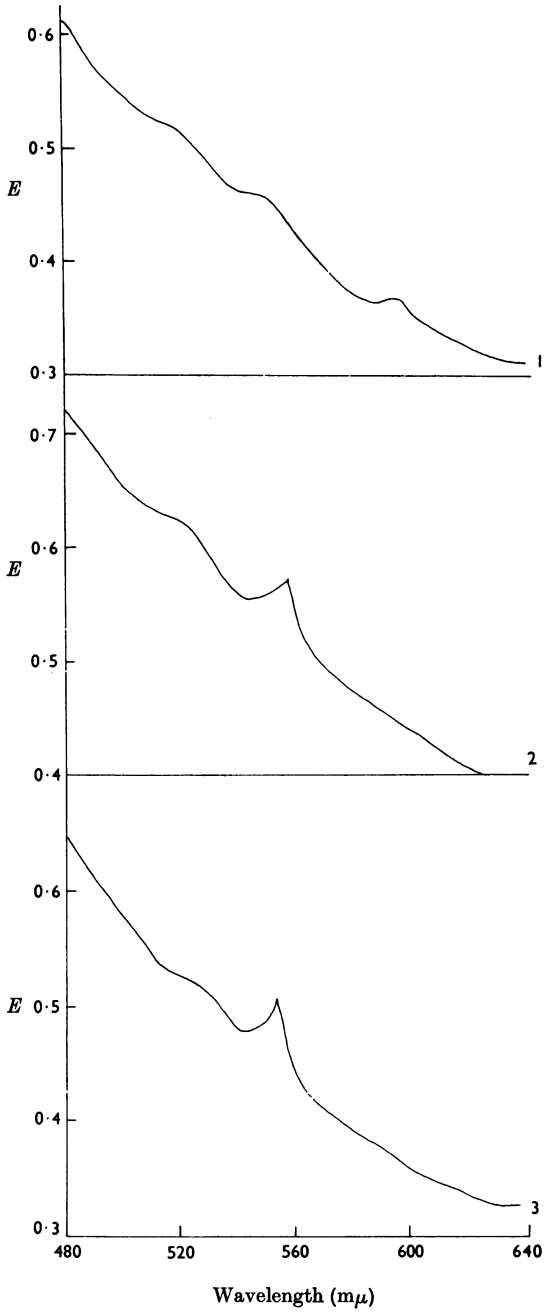


Fig. 2. Absorption spectra of cytochromes in solubilized subcellular fractions from the bovine adrenal medulla from 480 to 620 mμ, measured in a Zeiss PMQIII spectrophotometer, 1 cm. path length. Curve 1: material from the upper band reduced with sodium dithionite. Curve 2: material from the lower band reduced with sodium dithionite. Curve 3: microsomal material reduced with sodium dithionite.

(Fig. 2). For the microsomes and lower band these were at 558 and 526mμ, the former being much the more intense. When the material from the upper band was reduced peaks appeared at 552 and 600mμ. The peak at 552mμ is characteristic of cytochrome *c*, and that at 600mμ is probably caused by cytochromes *a* and a_3.

All these findings are consistent with the view that the upper band consists of mitochondria. On the other hand, the cytochrome pigment in the lower band was similar to that of the microsomes and is identified as cytochrome b_5: this is in agreement with an observation of Spiro & Ball (1958), who found a microsomal type of cytochrome in the chromaffin granules.

Distribution of ATPase activity between subfractions of the adrenal medulla. The ATPase activity of the upper and lower bands derived from the large granules and of the microsomes was measured in the presence of either 2·5mM-Mg^{2+} or 2·5mM-Ca^{2+}. Activity was found in each of the fractions (Table 2). The material from the upper band (mitochondria) and from the microsomes contained much higher activities than the material from the lower, catecholamine-rich, band; this was not only true when the activities were expressed in terms of mg. of protein nitrogen but also when they were referred to as fresh weight of tissue (Table 3). Table 2 also shows that the material from the microsomes was more active in the presence of 2·5mM-Ca^{2+} than in the presence of 2·5mM-Mg^{2+}.

The granules in the lower band were disrupted by osmotic shock and the water-soluble and water-insoluble protein were tested separately for ATPase activity. In an experiment in which 32·6% of the total protein of the fraction was recovered in the insoluble material, the specific activity of the ATPase in the insoluble protein was 39·7 μmoles of inorganic phosphate liberated/mg. of protein nitrogen/hr.: no ATPase activity was detected in the soluble protein fraction.

Further resolution of the chromaffin granules. The presence of large amounts of catecholamines and ATP in the lower band obtained by density-gradient centrifugation indicates that the fraction contains chromaffin granules. Further, the absence of succinate dehydrogenase and mitochondrial cytochromes suggests that mitochondria cannot be responsible for the ATPase activity of the fraction. However, contamination of the lower band by microsomal particles could possibly account for the presence of ATPase in the catecholamine-rich fraction and for the finding that the cytochrome pigments in the lower band and the microsomes are identical. To reduce the risk of contamination with microsomes, the large granules were sedimented at 5000*g* for 20min. instead of 11000*g* for 20min. After subfractionation of the resuspended large granules by density-gradient centrifugation, the lower band showed a distinct separation into two layers. The top layer, A, was grey–white, whereas the bottom layer, B, was somewhat pink, wider and more dense than layer A (Fig. 3). Table 4 shows that layer B contained more catecholamines and ATP/mg. of protein nitrogen than did layer A. About one-third of the protein recovered in the lower

Table 2. *Specific activity of ATPase in adrenal medullary subfractions*

Experimental details are given in the text. The values obtained in the presence of Mg^{2+} are the means of five experiments and those in the presence of Ca^{2+} are the means of three experiments.

	Specific activity of ATPase (μmoles of P_i liberated/mg. of protein N/hr.)		
Addition	Upper band	Lower band	Microsomes
Mg^{2+} (2·5mM)	47·1	11·7	59·1
Ca^{2+} (2·5mM)	42·1	10·7	90·4

Table 3. *Total ATPase activity of subfractions from the bovine adrenal medulla in the presence of* Mg^{2+} (2·5*m*M)

Experimental details are given in the text. The values obtained for the upper band and the lower band are the means of five experiments and that for microsomes is the mean of four experiments.

Total ATPase activity (μmoles of P_i liberated/g. wet wt. of tissue/hr.)		
Upper band	Lower band	Microsomes
35·5	5·0	52·9

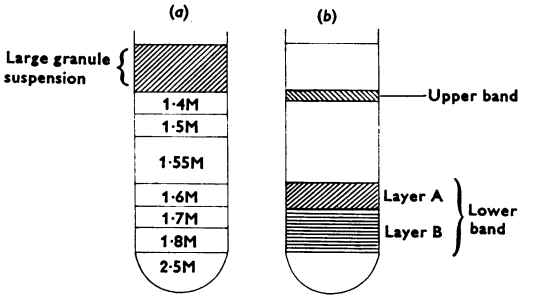


Fig. 3. Composition of sucrose density gradient and distribution of material after density-gradient centrifugation. (*a*) Composition of a sucrose density gradient before centrifugation; the molarities refer to sucrose. (*b*) Distribution of material after centrifugation at 145000*g* for 1hr.

Table 4. *Distribution of catecholamines, ATP and protein nitrogen between layers A and B of the lower band of material obtained when the large granules were submitted to density-gradient centrifugation, after preliminary high-speed centrifugation at 5000g for 20min.*

Experimental details are given in the text.

	Catecholamines (μmoles)	ATP (μmoles)	Protein N (mg.)	Catecholamine / ATP molar ratio	Catecholamines (μmoles/mg. of protein N)
Layer A	2·91	0·44	0·35	6·6	8·3
Layer B	10·70	2·23	0·75	4·8	14·3

band was in layer A but only about one-fifth of both the catecholamines and ATP was recovered in that layer. The cytochrome pigment of layer B resembled that found in both the microsomes and the lower band in the previous experiments (see Figs. 1 and 2).

Electron microscopy. Plate 1 shows electron micrographs made from the upper band, the two layers of the lower band and the microsomes respectively. The upper band consists of swollen mitochondria with the internal membranes highly vacuolated (Plate 1*a*). Layer A is shown in Plate 1(*b*); this layer contains both mitochondria and densely osmiophilic granules of differing diameters that are strongly reminiscent of the chromaffin granules seen in intact adrenal medullary cells. Layer B contains these granules as the only well-defined particles and has no obvious admixture of mitochondria (Plate 1*c*). Layer A also contains some elements that appear to be derived from the endoplasmic reticulum; such profiles are absent from Layer B. In many instances the chromaffin granules can be seen to possess an outer membrane to which some fluffy material usually adheres. In addition to granules with circular profiles, both layers A and B contain tubular elements with densely osmiophilic cores and a membrane. Since these tubular elements have not been seen in sections of intact cells they are probably artifacts produced by mechanical distortion of the chromaffin granules. Similar elongated particles have been seen by Hagen & Barrnett (1960) in a preparation of chromaffin granules. Plate 1(*d*) shows a section through the microsomal pellet. There are no obvious mitochondrial or chromaffin-granule profiles present; the chief feature is a mass of fragmented membranes and vesicles.

ATPase activity of the chromaffin granules. To find out if the purified preparation of chromaffin granules contained ATPase activity, the insoluble material from layers B (pure chromaffin granules) and A (chromaffin granules contaminated by mitochondria) was prepared and tested in the presence of different concentrations of Ca^{2+} and Mg^{2+}. The results of these experiments and of

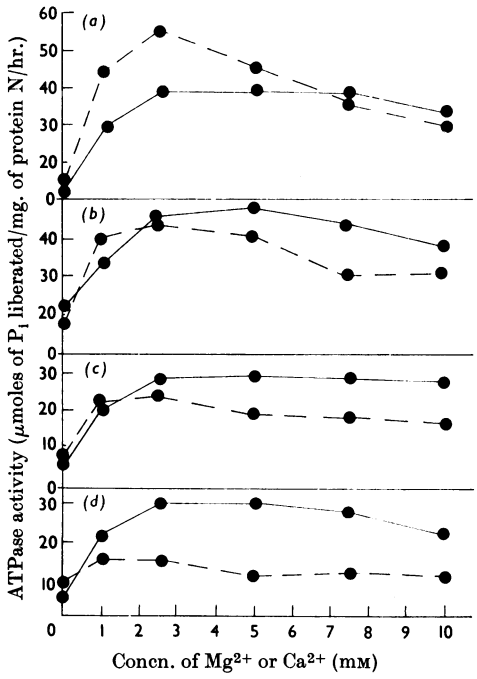
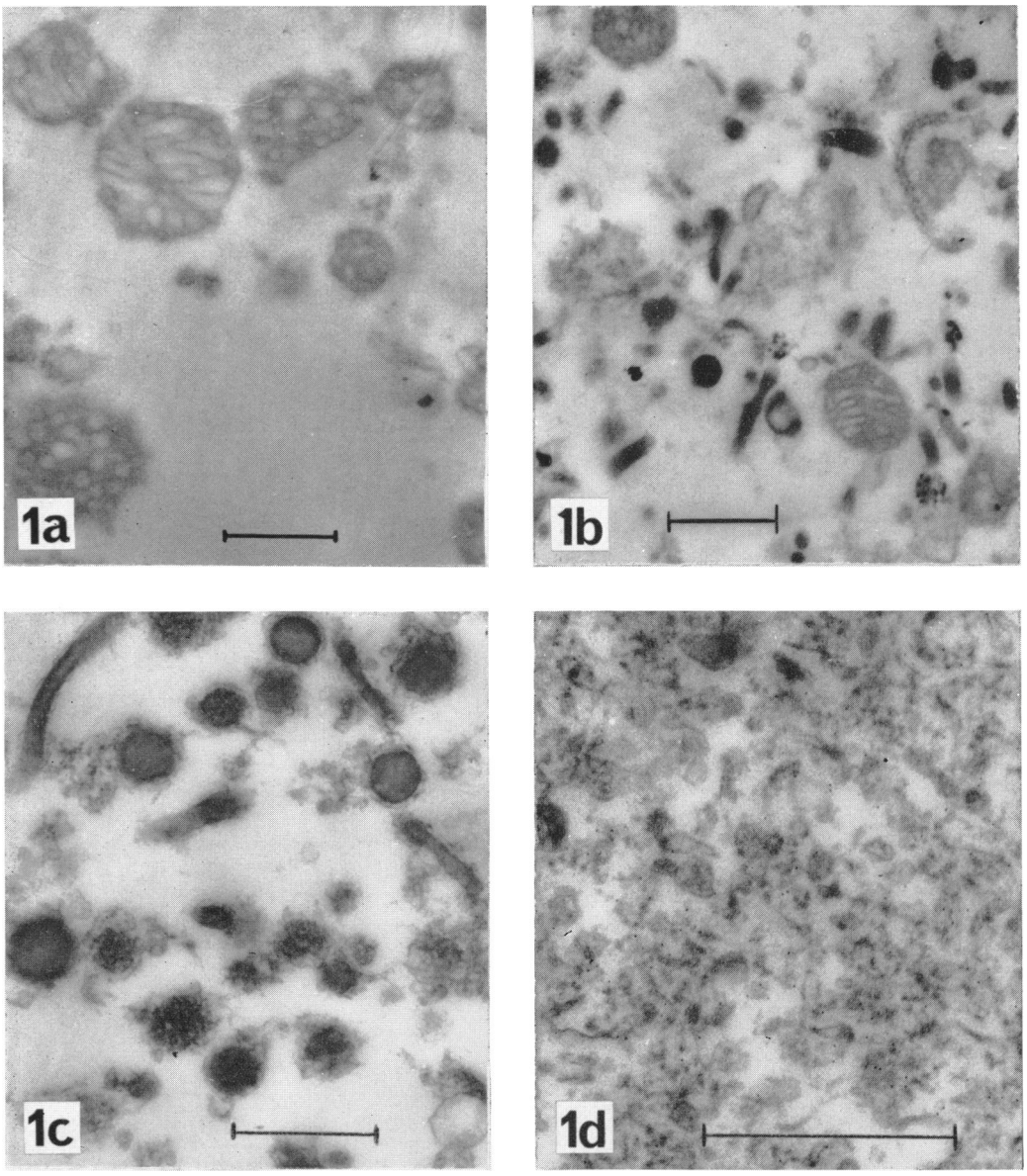


Fig. 4. Effect of Mg^{2+} (——) and Ca^{2+} (– – – –) on the ATPase activities of fractions from the bovine adrenal medulla at 37°. The reaction mixture contained: ATP (5mM), KCl (20mM) and tris–HCl buffer (33mM), pH 7·4. (*a*) Microsomal fraction; (*b*) upper band; (*c*) layer A of lower band; (*d*) layer B of lower band.

similar observations on the microsomes and on the upper band (mitochondria) are shown in Fig. 4. All the fractions had ATPase activity. The microsomal enzyme was stimulated more by Ca^{2+} ions than by Mg^{2+} ions over the concentration range 1–5mM, although the two ions had similar activating effects over the range 5–10mM. The mitochondrial ATPase was stimulated to roughly the same extent by Ca^{2+} and Mg^{2+} ions over the range 1–3mM, but above 3mM Mg^{2+} was more effective than Ca^{2+}. In contrast, the ATPase activity of layer B showed a



EXPLANATION OF PLATE 1

Electron micrographs of fractions from bovine adrenal medulla: (*a*) upper band (magnification 29000×); (*b*) layer A of the lower band (magnification 29000×); (*c*) layer B of the lower band (magnification 40000×); (*d*) microsomal fraction (magnification 67000×). In each photograph the scale represents 0·5 μ.

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much more conspicuous response to the addition of Mg^{2+} than of Ca^{2+} over the whole range of concentrations used. These observations strongly suggest that the ATPase activity in the purified preparation of chromaffin granules (layer B) is not due to contamination by either mitochondrial or microsomal elements. The results obtained with the material from layer A were intermediate between those obtained for the mitochondria and layer B. This agrees well with the composition of the fraction, which the electron micrographs showed to be a mixture of the two kinds of particle.

To prove that the enzyme present in the chromaffin granules was an ATPase, the nucleotide reaction products obtained after incubation of the insoluble material from layer B with ATP were isolated and separated by ion-exchange chromatography on Dowex AG-1 resin and found to be ADP. The amount of ADP produced was equal to the amount of inorganic phosphate liberated. Na^+ (150mM) and K^+ (40mM) added together did not stimulate the ATPase activity of the microsomes, or of the upper and lower layers prepared from the large granules sedimented at 11000*g*. Indeed, the presence of 150mM-Na^+ inhibited the microsomal and mitochondrial ATPases.

DISCUSSION

The chromaffin cells of the bovine adrenal medulla contain active ATPases in their mitochondria and microsomes. To decide whether or not the chromaffin granules have an intrinsic ATPase it was essential to obtain a preparation of these granules free from mitochondrial or microsomal contamination. The electron micrographs show that this aim has been achieved.

An ATPase activity has been demonstrated in a fraction whose sedimentation characteristics, content of catecholamines and molar ratio of catecholamines to ATP identify it as being composed of chromaffin granules. The inability to demonstrate succinate-dehydrogenase activity in the fraction indicated that contamination by mitochondria was very low: this was supported by the absence of typical mitochondrial cytochromes from the fraction. However, when further purification allowed the band of chromaffin granules to be divided into two subfractions, one of them was found to contain some mitochondria. This contamination was insufficient to cause demonstrable succinate-dehydrogenase activity or to allow the identification of mitochondrial cytochromes in the unresolved chromaffin-granule fraction (lower band). In the most highly purified preparation (layer B of the lower band) chromaffin granules were the only well-defined particles. This fraction still retained an enzyme, activated by Mg^{2+}, that degrades ATP to ADP and inorganic phosphate. The cytochrome pigment in the chromaffin-granule fraction is spectroscopically identical with that found in the microsomes, but the activating effects of Mg^{2+} and Ca^{2+} on the ATPases of the two fractions are distinct. This evidence indicates that the ATPase activity found in a purified preparation of chromaffin granules is different from those in the mitochondria and microsomes and cannot be attributed to contamination by mitochondrial or microsomal particles. This conclusion supports the claim of Hillarp (1958*b*) that the chromaffin granules have an intrinsic ATPase activity.

The total activity of the ATPase of the chromaffin granules is much lower than that of the mitochondria or microsomes. This low activity may help to identify the physiological role of the ATPase. A Mg^{2+}- and ATP-dependent uptake of catecholamines by isolated chromaffin granules against a concentration gradient has been described by Kirshner (1962*a*,*b*) and by Carlsson, Hillarp & Waldeck (1962, 1963). Kirshner (1962*b*) showed that the uptake is endothermic, suggesting that an active process is involved in amine accumulation. This is in contrast with the maintenance of high concentrations of catecholamines in the granules, which is not dependent on a supply of energy (Hillarp & Nilson, 1954). The rate of biosynthesis is low; e.g. Udenfriend, Cooper, Clark & Baer (1953) found the half-life of adrenal catecholamines to be 9 days in rats. This suggests that the ATPase activity of the chromaffin granules is related to their ability to take up catecholamines, and that its low activity may be sufficient to remove the amines from the cytoplasm after their formation. The low activity of the enzyme and the small activating effect of Ca^{2+}, an ion thought to act as the secretion–stimulus coupling agent (Douglas & Rubin, 1961; Schümann & Philippu, 1962), render it unlikely that the ATPase activity of the chromaffin granules is involved in the secretion of catecholamines.

The similarity between the cytochrome pigment of the chromaffin granules and that of the microsomes may be a reflection of the origin of the granules in the Golgi region of the endoplasmic reticulum, as proposed by de Robertis & Sabatini (1960).

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REFERENCES

Blaschko, H., Born, G. V. R., D'Iorio, A. & Eade, N. R. (1956). *J. Physiol.* **133**, 548.

Blaschko, H., Hagen, J. M. & Hagen, P. (1957). *J. Physiol.* **139**, 316.

Carlsson, A., Hillarp, N. Å. & Hökfelt, B. (1957). *J. biol. Chem.* **227**, 243.

Carlsson, A., Hillarp, N. Å. & Waldeck, B. (1962). *Med. exp.* **6**, 47.

Carlsson, A., Hillarp, N. Å. & Waldeck, B. (1963). *Acta physiol. scand.* **59** (suppl.), 215.

de Robertis, E. D. P. & Sabatini, D. D. (1960). *Fed. Proc.* **19**, (suppl. 5), 70.

Douglas, W. W. & Rubin, R. P. (1961). *J. Physiol.* **159**, 40.

Euler, U. S. von & Hamberg, U. (1949). *Acta physiol. scand.* **19**, 74.

Fortier, A., Leduc, J. & D'Iorio, A. (1959). *Rev. Canad. Biol.* **18**, 110.

Hagen, P. (1962). *Biochem. J.* **82**, 25P.

Hagen, P. & Barrnett, R. J. (1960). *Ciba Found. Symp.*: *Adrenergic Mechanisms*, p. 83. Ed. by Vane, J. R., Wolstenholme, G. E. W. & O'Connor, M. London: J. and A. Churchill Ltd.

Hillarp, N. Å. (1958*a*). *Acta physiol. scand.* **42**, 144.

Hillarp, N. Å. (1958*b*). *Acta physiol. scand.* **43**, 82.

Hillarp, N. Å. & Nilson, B. (1954). *Acta physiol. scand.* **31** (suppl.), 113.

Kirshner, N. (1962*a*). *Science*, **135**, 107.

Kirshner, N. (1962*b*). *J. biol. Chem.* **237**, 2311.

Kuff, E. L. & Schneider, W. C. (1954). *J. biol. Chem.* **206**, 667.

LePage, G. A. (1957). In *Manometric Techniques*, p. 268. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.

Schümann, H. J. (1957). *J. Physiol.* **137**, 318.

Schümann, H. J. (1958). *Arch. exp. Path. Pharmak.* **233**, 237.

Schümann, H. J. & Philippu, A. (1962). *Experientia*, **18**, 138.

Spiro, M. J. & Ball, E. G. (1958). *Fed. Proc.* **17**, 314.

Strehler, B. W. & Totter, J. R. (1954). *Meth. biochem. Anal.* **1**, 341.

Udenfriend, S., Cooper, J. R., Clark, C. T. & Baer, J. F (1953). *Science*, **117**, 663.