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MITOCHONDRIAL ENZYMES AND CHROMAFFIN GRANULES

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In a homogenate of the bovine adrenal medulla in isotonic sucrose the catechol amines are present mainly in cytoplasmic granules; these granules are sedimented in a gravitational field similar to that used for the sedimentation of mitochondria in other tissues (Blaschko & Welch, 1953). Preparations of the adrenal granules were found to oxidize substrates of the tricarboxylic acid cycle (Blaschko, Hagen & Welch, 1955), but the question whether or not the amines were carried in the same granules as the respiratory enzymes remained unanswered. It was noted in these experiments that the high-speed sediment could be subdivided into a 'top' and a 'bottom' layer; the top fraction was richer in succinoxidase, and poorer in amines, than the bottom fraction. This observation suggested the possibility that there might be two different types of granule, which had been imperfectly separated by the method employed.

It has since been found that granules with a high amine content can be sedimented in strongly hypertonic sucrose (Blaschko, Born, D'Iorio & Eade, 1956). In one of the experiments reported, a suspension of granules prepared in isotonic 0·3 M sucrose was centrifuged over 1·5 M sucrose: granules were retained at the boundary region between the isotonic and the hypertonic sucrose; these granules contained very little catechol amine.

In the present work suspensions of 'large granules' from the bovine adrenal medulla in isotonic sucrose were centrifuged over a specific gravity gradient. Different fractions were thus obtained, and these were examined for catechol amines, for adenosine triphosphate (ATP) and for a number of enzymes normally present in mitochondria.

METHODS

Preparation of 'large granules'. Ox adrenal medulla was chopped and homogenized in 0·3 M sucrose; the homogenate was centrifuged at low speed (950 *g*) for 20 min. The low-speed supernatant, centrifuged at 11,000 *g* for 30 min, gave a sediment which was resuspended in 0·3 M sucrose and recentrifuged at 11,000 *g* for 30 min. The sediment was again suspended in 0·3 M

sucrose, and this suspension of 'large granules' was used as the starting material for the specific gravity gradient centrifugation.

Preparation of the specific gravity gradient tubes. The three tubes of the SPINCO swing-out rotor SW39L were prepared by layering 1 ml. each of sucrose solutions in the order: 2·25M, 2·0M, 1·8M, 1·6M, with the most concentrated solution at the bottom of the tube. The tubes were prepared a few hours before use. In each tube 1 ml. of the large-granule suspension was layered above the 1·6M sucrose at the top of the gradient (see Fig. 1*a*). The tubes were then centrifuged at 145,000 *g* for 1 hr. At the end of the run the positions of the layers formed were noted, and the tubes were cut. (The cutter was kindly presented to us by Dr E. H. Schuster.) The fractions collected were then frozen and kept at about −12° C until the determinations were carried out; these were always completed within 48 hr of collection.

Determination of catechol amines, ATP and respiratory enzymes. In all experiments the catechol amines were determined by the method of von Euler & Hamberg (1949), but with the buffer solutions used by Schümann (1957). In two experiments ATP was determined by the firefly luminescence method.

Succinic dehydrogenase was estimated spectrophotometrically, by measuring the reduction of cytochrome c at 550 mμ in the presence of cyanide, as described by Kuff & Schneider (1954). Fumarase was determined according to Racker (1950), by measuring the increase in optical density at 240 mμ when malate is reduced to fumarate. Amine oxidase was determined manometrically, by following the oxygen uptake with 0·01M tyramine as substrate in the presence of 0·01M semicarbazide.

Protein was precipitated by adding 10% trichloroacetic acid to each sample. The precipitate was spun down, resuspended in trichloroacetic acid and again sedimented. The nitrogen content of the sediment was determined after digestion in a micro-Kjeldahl flask and steam distillation.

RESULTS

The specific gravity gradient was prepared as is shown in Fig. 1*a*; the typical appearance of the tube, at the end of the centrifugation at 145,000 *g* for 1 hr in a horizontal rotor, is shown in Fig. 1*b*. In some of the experiments no sediment was seen, but in others a very small amount of whitish material had sedimented. Several opaque layers had formed at the boundaries of the sucrose solutions. One well-defined opaque band was always seen at the junction of the 2·0M and the 2·25M sucrose, and another at the top of the gradient, between the 0·3M and the 1·6M sucrose. A layer was also always present between the 1·8M and the 2·0M sucrose solutions. The band between the 1·6M and the 1·8M sucrose was not seen in all experiments.

Fig. 1*b* also shows the positions at which the tubes were cut for the collection of the fractions. The different fractions collected were numbered as shown in the figure.

Figs. 2–5 give the results obtained in some of these experiments. In these figures the percentages are calculated in terms of the amounts recovered in all the fractions. In the legends to the figures percentage recoveries are also given on the basis of the material present in the starting material, the large-granule suspension.

Fig. 2 shows an experiment in which succinic dehydrogenase, catechol amines and protein nitrogen were determined. The curve for protein nitrogen shows two maxima, one in fraction 2 and another in fraction 5. Succinic

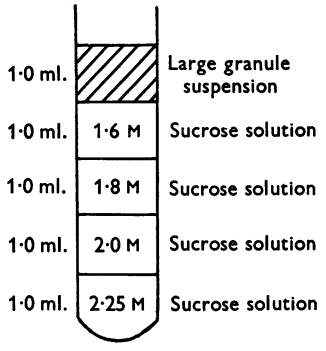

Fig. 1*a*

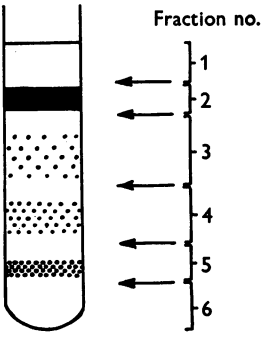

Fig. 1*b*

Fig. 1*a*. Centrifuge tube before specific gravity gradient centrifugation. The large granule fraction was suspended in 0·3 M sucrose solution.

Fig. 1*b*. Appearance of the tube after centrifugation in Spinco rotor SW 39 at 145,000 *g* for 60 min. The arrows indicate the points where the tube was cut.

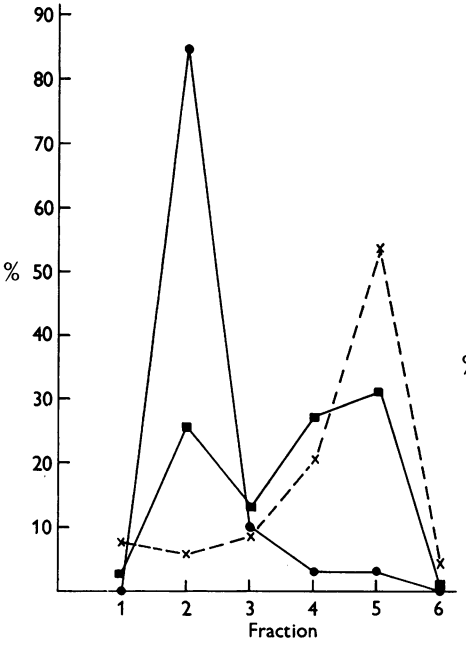

Fig. 2

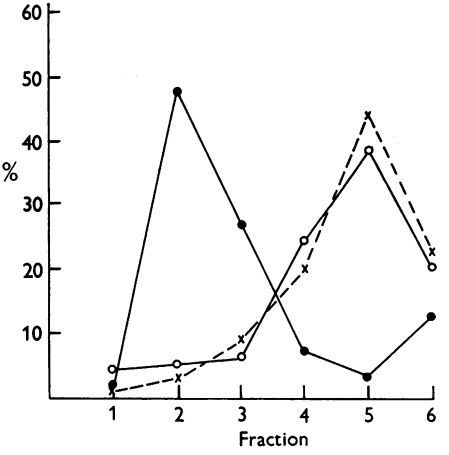

Fig. 3

Fig. 2. Distribution of succinic dehydrogenase (●—●), catechol amines (×- - -×) and protein nitrogen (■—■) in a specific gravity gradient. In Figs. 2–5 the fractions are numbered as shown in Fig. 1*b*. Ordinate: Recovery as percentage of recovery in all fractions. Total recoveries were; catechol amines 80%, succinic dehydrogenase 103%, protein nitrogen 60%.

Fig. 3. Distribution of succinic dehydrogenase (●—●), catechol amines (×- - -×) and ATP (○—○) in a specific gravity gradient. Ordinate: recovery as percentage of recovery in all fractions. Total recoveries; succinic dehydrogenase 80%, catechol amines 86%, ATP 54%.

dehydrogenase activity had a maximum in fraction 2; this fraction contained 84% of the activity recovered in all fractions. The pressor amines were present mainly in fraction 5, with 54% of the amount recovered in all fractions.

In the experiment shown in Fig. 3, determinations were carried out of succinic dehydrogenase, catechol amines and ATP. In this experiment succinic dehydrogenase was mainly distributed over fractions 2 and 3. The highest amine content was again found in fraction 5, but in this experiment some sediment had formed and this also contained some of the catechol amine. The figure shows that the ATP had a distribution very similar to that of the catechol amines. This finding was confirmed in a second experiment in which determinations of ATP were carried out.

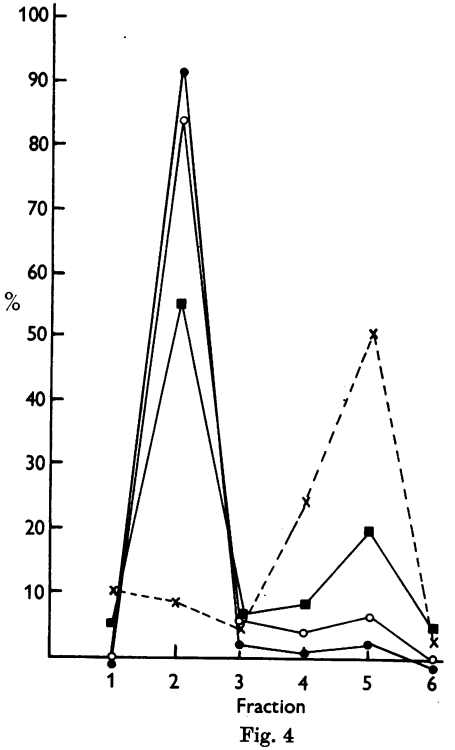

Fig. 4

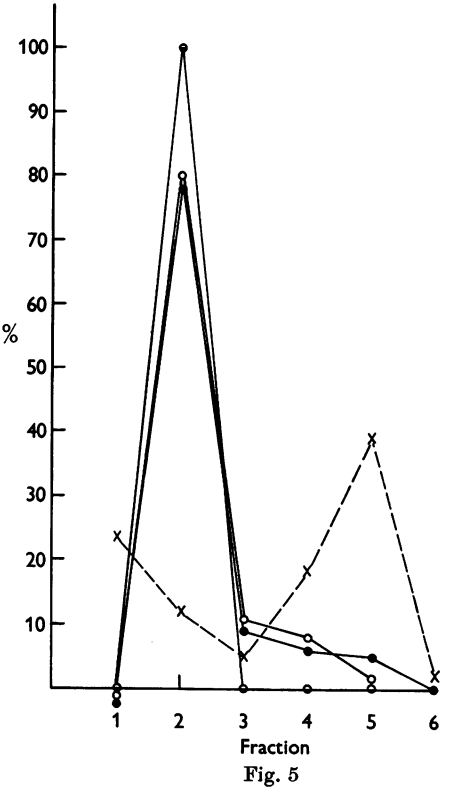

Fig. 5

Fig. 4. Distribution of succinic dehydrogenase (●—●), fumarase (○—○), catechol amines (× - - - ×) and protein nitrogen (■—■) in a specific gravity gradient. Ordinate: recovery as percentage of recovery in all fractions. Total recoveries; succinic dehydrogenase 136%, fumarase 111%, catechol amines 96%, protein nitrogen 61%.

Fig. 5. Distribution of succinic dehydrogenase (●—●), fumarase (○—○), amine oxidase (◒—◒) and catechol amines (× - - - ×) in a specific gradient. Ordinate: recovery as percentage of recovery in all fractions. Total recoveries; succinic dehydrogenase 87%, fumarase 76%, amine oxidase 55%, catechol amines 75%.

The experiment shown in Fig. 4 is one in which fumarase activity was also determined in the fractions. Again there was a single peak for catechol amines in fraction 5 and two peaks for protein nitrogen, one in fraction 2 and the other in fraction 5. The two enzymes, succinic dehydrogenase and fumarase, each had a sharp maximum in fraction 2.

Amine oxidase activity, in addition to succinic dehydrogenase and fumarase, was determined in the experiment of Fig. 5. Amine oxidase activity was recovered only in fraction 2; this fraction also contained the bulk of the two other enzymes. In this experiment some catechol amine was also found in the two uppermost fractions, 1 and 2; this represents amine released from the granules in the course of the experiment and now present in free aqueous solution.

Eade (1956) has reported that upon centrifugation of a suspension of granules from the bovine adrenal medulla over hypertonic sucrose the adrenaline and the noradrenaline are differently distributed. Similar differences were found in the present series of experiments. In seven experiments, the combined fractions 3+4, contained 39% of the adrenaline recovered, but only 19% of the noradrenaline. The amount of noradrenaline recovered in fractions 5+6 was correspondingly greater: 59% as compared with 43% of the adrenaline.

DISCUSSION

By the use of conventional histological techniques, mitochondria have been detected in the chromaffin cells of a number of species (Bennett, 1941; Hillarp, Hökfelt & Nilson, 1954); however, the latter authors were not able to see them in the adrenal medulla of the cow. It seems likely that the bovine material was less fresh than that from the laboratory animals examined.

The histological studies have recently been supplemented by observations under the electron microscope. Typical mitochondria, with outer membranes and cristae, have been seen in the chromaffin cells of the rat (Lever, 1955), the mouse, the guinea-pig and the cat (Sjöstrand & Wetzstein, 1956).

In their study of the distribution of succinic dehydrogenase in mouse liver Kuff & Schneider (1954) used a specific gravity gradient; they found that the bulk of the enzymic activity was recovered above a layer of 1·59 M sucrose. It seems, therefore, that in our experiments the layers which were rich in respiratory enzymes had physical properties similar to liver mitochondria. This, therefore, seems to be the true 'mitochondrial' fraction. That in the chromaffin tissue the amine oxidase is also found in this fraction is interesting for two reasons, first, because it shows that the catechol amines are mainly stored in a cytoplasmic structure different from that which contains amine oxidase, and secondly, because it confirms by a different method earlier observations on the localization of this enzyme in liver cells (Cotzias & Dole, 1951; Hawkins, 1952).

Under the electron microscope a second type of granule can be distinguished

in the chromaffin cell; this is characterized by its strongly osmiophilic properties (Lever, 1955; Sjöstrand & Wetzstein, 1956). It is likely that this is the granule described by Hillarp *et al.* (1954) in smears of chromaffin tissue. Osmic acid is a histological reagent for the demonstration of the amine in the adrenal medulla. It seems likely, therefore, that the osmiophilic granules are those which settled in the lower fractions of the specific gravity gradient in our experiments. It is interesting that in the electron-microscopic study these granules were found to possess an outer membrane. It is this membrane which is probably responsible for the fact that the catechol amines present in a suspension of fresh granules in isotonic sucrose, injected intravenously into the spinal cat, do not immediately exert their full biological effect (Blaschko *et al.* 1955).

The experiments show that the ATP and the catechol amines are very similarly distributed throughout the different fractions. This is in support of the view that the two substances are present in one and the same granule and that ATP is closely connected with the storage of the catechol amines (Falck, Hillarp & Högberg, 1956; Blaschko *et al.* 1956).

In earlier experiments, in which the suspending medium was isotonic sucrose, it was not possible to separate the mitochondria from the chromaffin granules. Electron microscopy has shown that the osmiophilic granules are not of uniform size, and this is probably why they do not sediment as a well-defined separate layer in isotonic sucrose. In the specific gravity gradient, however, the position of a particle after centrifugation is not determined by its size but by its specific gravity. This made separation under the conditions now used more successful.

Hillarp *et al.* (1954) have discussed the possibility that in the chromaffin cell the amine-carrying granules take the place of true mitochondria. In our experiments, a little enzymic activity was usually found in the lower layers of the gradient tube. It can therefore not be entirely excluded that there exists a relationship between mitochondria and chromaffin granules. However, the electron microscope as well as our experiments agree in revealing the presence of two different types of granule. It seems, therefore, at present safer to assume that the two structures are distinct.

SUMMARY

1. Suspensions of large granules from the bovine adrenal medulla in isotonic sucrose have been prepared; these suspensions have been centrifuged at high speed over a specific gravity gradient.

2. The different fractions thus obtained have been examined for catechol amines and ATP as well as for succinic dehydrogenase, fumarase and amine oxidase.

3. The major part of the three enzymes was found in the upper fractions

of the gradient; catechol amines and ATP were present mainly in the lower fractions.

4. It is concluded that mitochondria and chromaffin granules are different cytoplasmic particles.

We are grateful to Dr W. D. McElroy for giving us the fireflies used in this work, and to Dr G. V. R. Born for placing his apparatus for the ATP determinations at our disposal.

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