J. Physiol. (1966), **183**, *pp.* 179–188
With 3 text-figures
Printed in Great Britain

THE LOCALIZATION OF LYSOSOMAL ENZYMES IN CHROMAFFIN TISSUE

BY A. D. SMITH AND H. WINKLER
From the Department of Pharmacology, University of Oxford

(*Received* 12 *August* 1965)

SUMMARY

1. An homogenate of bovine adrenal medulla contains significant amounts of six acid hydrolases: acid ribonuclease, acid deoxyribonuclease, cathepsin, acid phosphatase, β-glucuronidase and arylsulphatase. Most of the activity of each enzyme could be sedimented in the large-granule fraction at 242,000 g-min.

2. Differential centrifugation indicated the presence of three populations of particles, which sedimented at slightly different rates; these are, in order of decreasing sedimentation rate, mitochondria, particles containing the acid hydrolases, and chromaffin granules.

3. The three types of particle could be separated by ultracentrifuging the large-granule fraction in a sucrose density gradient. Most of the activity of each hydrolase was recovered in a layer intermediate between those formed by mitochondria and chromaffin granules.

4. The large-granule fraction therefore contains particles which are defined by their enzyme content as lysosomes.

5. Highly purified chromaffin granules, containing less than 5% of the activity of each acid hydrolase, were obtained from the gradient.

INTRODUCTION

A particulate fraction of bovine adrenal medulla which contains the catecholamines has been shown to possess ribonuclease activity. It has been suggested that this enzyme may be involved in the spontaneous release of catecholamines and adenosinetriphosphate from isolated chromaffin granules (Philippu & Schümann, 1964).

We have recently found that, in addition to ribonuclease, a 'chromaffin granule fraction' contained deoxyribonuclease. A study of these enzymes after partial purification showed that they had acid pH optima and other properties in common with the acid nucleases of rat liver lysosomes (Smith & Winkler, 1965).

Rat liver lysosomes are denser than mitochondria when centrifuged in a

sucrose gradient (Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959) and the same is true of adrenal chromaffin granules (Blaschko, Hagen & Hagen, 1957). The question arose whether the chromaffin granule fraction was contaminated with lysosomes. We have therefore studied the distribution of six typical lysosomal enzymes, namely acid ribonuclease, acid deoxyribonuclease, acid phosphatase, cathepsin, β-glucuronidase and arylsulphatase, between different particulate fractions obtained from homogenates of bovine adrenal medulla. A partial separation of the enzymes β-glucuronidase and acid phosphatase from catecholamine-containing granules has recently been reported (Koenig, Mylroie, Gaines, Gray & McDonald, 1965).

METHODS

Preparation and centrifugation of homogenate. Ox adrenal medullae were finely chopped and homogenized in a Potter–Elvehjem homogenizer (clearance 0·08 mm) having a glass mortar and Teflon pestle, to give a 1:5 (w/v) homogenate in 0·3 M sucrose. Homogenization was carried out as gently as possible, by passing the pestle up and down no more than three times.

In the centrifugal data given below, all the values for g were calculated using the radius from the centre of rotation to the bottom of the tube. The centrifugal force is given as g times minutes (g-min).

The homogenate was centrifuged at low speed (9×10^3 g-min) to remove unbroken cells and cell nuclei. A large-granule fraction was obtained from the low-speed supernatant by centrifugation at $2{\cdot}42 \times 10^5$ g-min in the A 40 rotor of the Spinco model L ultracentrifuge at 2° C. The sediment was resuspended in 0·3 M sucrose so that 1 ml. of the resuspended large-granule fraction corresponded to approximately 0·7 g of the original tissue. The sucrose density gradients were prepared by layering with a pipette sucrose solutions of decreasing molarity one above another in a centrifuge tube. The gradient was composed of 0·25 ml. of 2·5 M sucrose and 0·5 ml. each of 2·0, 1·9, 1·8, 1·7, 1·6, 1·5, 1·4, 1·3 M sucrose. The tubes containing the completed gradients were kept at 3° C for 24 hr, and immediately before use 0·5 ml. of resuspended large-granule fraction was layered above the sucrose gradient (see Fig. 2*a*). The tubes were centrifuged at $87{\cdot}4 \times 10^5$ g-min in the Spinco swing-out rotor SW 39 L. The position of the bands after centrifugation was noted, and the tubes cut with the Schuster centrifuge tube cutter in the positions indicated in Fig. 2*b*. The fractions from three identical sucrose gradient tubes were pooled for analysis.

Determination of catecholamines and of enzyme activities. The catecholamines were measured by the method of von Euler & Hamberg (1949) but with citrate–phosphate buffers (McIlvaine, 1921) instead of acetate buffers. Protein nitrogen was estimated by the micro-Kjeldahl method. For enzyme assays the fractions were diluted with Tris/Na succinate buffer pH 5·9 (I = 0·015) in order to lyse the cytoplasmic particles. The diluted fractions were dialysed against the same buffer for 16 hr at 3° C in order to reduce the otherwise high enzyme blanks. Spectrophotometric assays were used for fumarase (Racker, 1950) and uricase (Schneider & Hogeboom, 1952). Acid phosphatase (β-glycerophosphatase) and β-glucuronidase were assayed by the methods of Gianetto & de Duve (1955). Cathepsin activity was measured by the method of Anson (1938), and arylsulphatase according to Roy (1956) using p-nitrophenylsulphate as substrate at pH 5·9.

For the assay of ribonuclease and deoxyribonuclease the optimum conditions of substrate concentration, pH and ionic strength were determined. Ribonuclease activity was estimated using a modification of the precipitation procedure of Roth & Milstein (1952). The enzyme, buffer (Tris/Na succinate pH 5·5, final I = 0·07) and substrate (yeast ribonucleic

acid, final concentration 0·2 mg/ml.), in a total volume of 2·5 ml., were incubated at 37° C for 1 hr; the ribonucleic acid was precipitated by adding 2·5 ml. of a solution containing ethanol, in a concentration of 50 % (v/v), and HCl (0·4 N). The tubes were kept at 0° C for 30 min before centrifuging, after which the optical density at 260 mμ of the supernatant was measured. This method of precipitation was found to be preferable to the zinc uranyl acetate method because the optical density of the uranyl acetate ion is so high. Deoxyribonuclease activity was measured by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) except that the acetate buffer had a final ionic strength of 0·136 and a pH of 4·6. The final substrate concentration was 0·2 mg/ml.

Units of enzyme activity are expressed by the method of de Duve *et al.* (1955) and refer to the transformation of 1 μmole of substrate/min/g of original tissue under the conditions of the assay, which was always carried out at 37° C. In the gradient experiments the units used were μmole of substrate/hr/ml. of fraction.

Chemicals. The following compounds were used as substrates for the enzyme assays: yeast ribonucleic acid and calf thymus deoxyribonucleic acid (British Drug Houses Ltd.); disodium DL-β-glycerophosphate, potassium *p*-nitrophenylsulphate, sodium L-malate and bovine haemoglobin (type 2) (Sigma London Chemical Co. Ltd.); sodium phenolphthalein-β-D-glucuronide (Koch-Light Laboratories Ltd.)

RESULTS

Acid hydrolases in the homogenate. The object of these experiments was to see whether the unresolved homogenate contained some of the acid hydrolases which are, in other tissues, largely confined to the lysosomes. Enzymic activities were determined after releasing any particle-bound activity by first lysing in a hypotonic buffer, and the results for six different enzymes are shown in Table 1. For comparison the table includes estimates of enzyme levels in rat liver homogenates made by other workers. The levels of acid ribonuclease, acid deoxyribonuclease and cathepsin were of the same order as those in rat liver.

TABLE 1. Acid hydrolase activities in homogenates. The figures are the means ± S.D. of total enzyme activities for three different adrenal medulla homogenates. The number of estimations is shown in parentheses. The mean values for rat liver are from Roy (1958) for arylsulphatase and from de Duve *et al.* (1955) for all other enzymes. The units are μmole substrate/min/g tissue

Enzyme	Bovine adrenal medulla	Rat liver
Acid ribonuclease	3·48 ± 0·5 (6)	2·7
Acid deoxyribonuclease	1·32 ± 0·03 (5)	1·3
Cathepsin	3·00 ± 0·08 (6)	1·5
Acid phosphatase	0·55 ± 0·08 (7)	6·0
β-Glucuronidase	0·05 ± 0·005 (6)	0·8
Arylsulphatase	0·06 ± 0·006 (4)	3·4

Differential centrifugation. Differential centrifugation in isotonic (0·3 M) sucrose was undertaken in order to compare the sedimentation properties of some of the particle-bound constituents. The low-speed supernatant was centrifuged in the swinging bucket rotor of an M.S.E. refrigerated centrifuge at 2° C. After centrifugation the supernatant was removed with a suction pipette, and the sediments were resuspended in

0·3 M sucrose. Sediments were obtained over a range of 20×10^3 to 130×10^3 g-min and the results of the assays were calculated as a percentage of the amount of the same material sedimented at $2{\cdot}52 \times 10^5$ g-min (Spinco ultracentrifuge, A 40 head). This value of g-min was chosen because no further sedimentation of the constituents used as markers for the particles could be obtained at higher values. The sedimentation properties

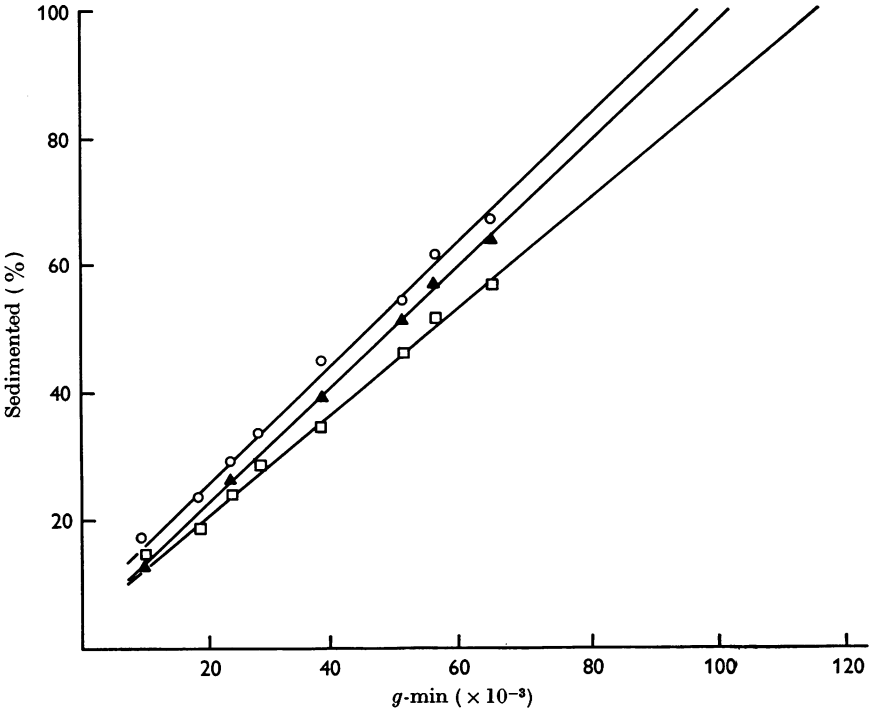

Fig. 1. Centrifugation of low-speed supernatant in 0·3 M sucrose. Each point represents the mean value of analyses from at least two sediments expressed as a percentage of the amount of sedimentable constituent. The lines were calculated by the method of least squares. ○, Fumarase; ▲, ribonuclease; □, catecholamines.

of the mitochondria (with fumarase as a marker) and the chromaffin granules (with catecholamines as a marker) were compared with those of the sedimentable acid ribonuclease. These experiments are illustrated in Fig. 1, which shows that fumarase sedimented most readily, catecholamines least readily, and that the behaviour of the ribonuclease activity was intermediate.

Sucrose density gradient centrifugation. The large-granule fraction (see Methods) contained 63 % of the catecholamines present in the low-speed supernatant; this percentage is of the same order as that found by earlier workers. The amount of the activity of the different acid hydrolases found

in the large-granule fraction ranged from 47% (acid phosphatase) to 81% (acid deoxyribonuclease) of that in the low-speed supernatant. No uricase activity was detected in the large-granule fraction.

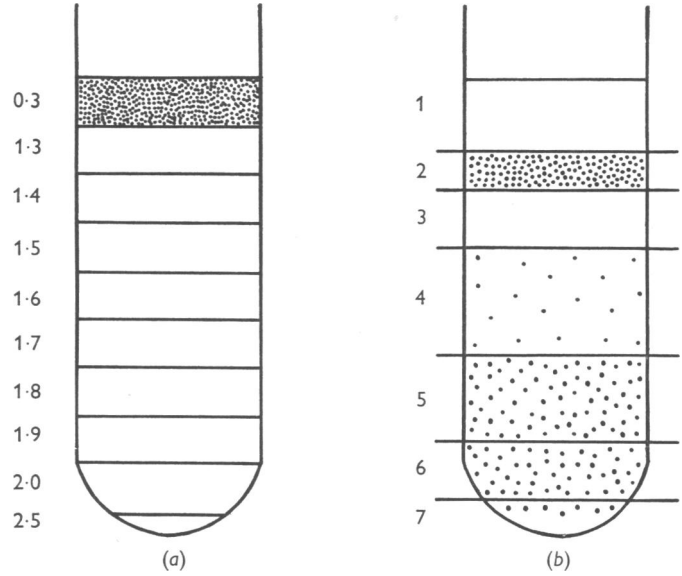

Fig. 2. Centrifugation of large-granule fraction in a sucrose gradient. (*a*) Composition of sucrose density gradient before centrifugation. Figures refer to the molarity of sucrose. (*b*) Distribution of material after centrifugation at $8{\cdot}74 \times 10^6$ *g*-min. The horizontal lines show the positions at which the centrifuge tube was cut: the different fractions are labelled 1 to 7.

The large-granule fraction was subjected to sucrose density gradient centrifugation as described under Methods (see also Fig. 2*a*). After centrifugation three distinct opaque regions were visible: fraction 2 was dark brown, 4 was light brown, 5 and 6 were light pink. This is illustrated in Fig. 2*b*. Altogether seven fractions were obtained from each gradient and these were each analysed for fumarase, protein nitrogen, catecholamines and six acid hydrolases. The recoveries ranged from 66 to 88%. This is satisfactory in view of the inevitable volume losses (*ca.* 10%) on cutting the tube and removing the fractions. The results of the analyses of the fractions from the sucrose gradients are shown in Fig. 3, where, in each histogram, the columns from left to right represent fractions from the top to the bottom of the centrifuge tube (see Fig. 2*b*). The presentation of the results of sucrose gradient experiments by means of histograms has been used previously by Beaufay, Jacques, Baudhuin, Sellinger, Berthet & de Duve (1964). Protein nitrogen (Fig. 3(*b*)), was concentrated in fractions 2, 5 and 6. For the other constituents three different types of pattern can be seen:

(1) Fumarase, a mitochondrial enzyme, was almost entirely confined to fraction 2 corresponding to an initial sucrose concentration of 1·3–1·4 M.

(2) Catecholamines were concentrated in fractions 5, 6 and 7 covering a range of initial sucrose concentrations from 1·8 to 2·0 M.

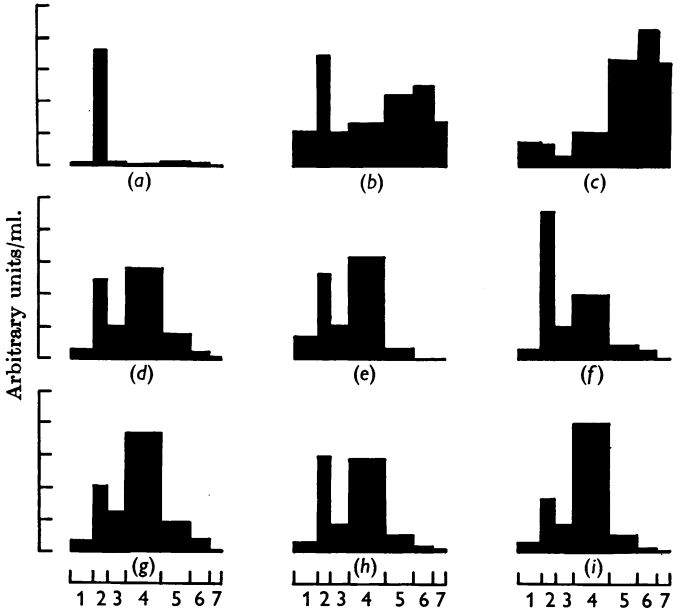

Fig. 3. Analysis of fractions from the sucrose gradient. The abscissa is divided according to the volumes of the fractions and the numbers refer to fractions of the gradient as shown in Fig. 2. The ordinates are arbitrary units per ml. and the actual value of one arbitrary unit is shown in parentheses below. The values for the enzymes are expressed in μmole substrate/hr/ml. of fraction. (*a*) Fumarase (43·6); (*b*) protein nitrogen (0·2 mg/ml.); (*c*) catecholamines (1·2 μmole/ml.); (*d*) acid ribonuclease (5·9); (*e*) cathepsin (3·5); (*f*) acid phosphatase (0·9); (*g*) acid deoxyribonuclease (2·9); (*h*) β-glucuronidase (0·1); (*i*) arylsulphatase (0·3).

(3) Each of the six acid hydrolases had a very similar bimodal distribution with one peak in fraction 2 and the other in fraction 4. In each case fraction 4 contained more enzyme than fraction 2, when the results were calculated as enzyme activity per whole fraction. The middle of fraction 4 corresponded to an initial sucrose concentration of 1·6 M.

DISCUSSION

Homogenates of bovine adrenal medulla contain significant amounts of six acid hydrolases. It is interesting to compare the specific activities with those found in rat liver by other workers. Cathepsin is twice as concentrated, ribonuclease and deoxyribonuclease are both present in similar

concentrations, but acid phosphatase, β-glucuronidase and arylsulphatase are less active than in rat liver. Histochemical evidence for the presence of acid phosphatase in the adrenal medulla of several species has been given by Eränkö (1952) and Novikoff (1961) and the presence of this enzyme has also been shown by biochemical methods (Hillarp & Falck, 1956). The latter authors suggested that acid phosphatase was present in the chromaffin granules of the ox adrenal medulla. An acid proteinase has previously been found in whole ox adrenal glands (Todd & Trikojus, 1960) at a concentration one-third of that found in the medulla in the present experiments. This suggests that the medulla contains most of the cathepsin activity of the gland. Arylsulphatase (Malmstrom & Glick, 1952) is also present in higher concentration in the medulla than in the cortex. A considerable proportion of the activity of each acid hydrolase in the homogenate was sedimented in our experiments, showing that the enzymes were associated with particles.

The results of differential and sucrose gradient centrifugation, to be discussed below, lead to the conclusion that the acid hydrolases of the bovine adrenal medulla are present in lysosomes. This is in agreement with the report of Koenig *et al.* (1965) already quoted. Biochemical and electron microscope studies of a human phaeochromocytoma (to be published) show that this tumour also contains lysosomes.

Differential centrifugation in isotonic sucrose was used to see whether it was possible to distinguish btween chromaffin granules, mitochondria and lysosomes. Acid ribonuclease activity was used as a marker for lysosomes, and the results shown in Fig. 1 indicate that this enzyme is present in a particle which sediments faster than chromaffin granules but slower than mitochondria. The lysosomes of rat liver also have a lower sedimentation rate in isotonic sucrose than the mitochondria (de Duve *et al.* 1955). The observation that, in 0·3 M sucrose, chromaffin granules sediment slower than mitochondria is what might be predicted from their small size in comparison with mitochondria (see the electron micrographs of Wetzstein, 1957). It is therefore interesting that in the experiments of Blaschko, Hagen & Welch (1955), Blaschko, Born, D'Iorio & Eade (1956) and Hillarp (1958) it was found that the amine-containing granules were concentrated in a pink layer at the bottom of the large granule sediment, and the particles now known to be mitochondria in a brown layer above the pink granules. The difference between these experiments and the present ones is that, in the earlier work, centrifugation was continued until all the sedimentable catecholamines had been spun down. Possibly, during prolonged centrifugation at high speed the particles are re-arranged in the sediment according to their density.

The results of differential centrifugation of the low-speed supernatant

show that there are only small differences between the sedimentation rates of the mitochondria, chromaffin granules and lysosomes. These results can be compared with the observations of Baudhuin, Beaufay, Rahman-Li, Sellinger, Wattiaux, Jacques & de Duve (1964) on rat liver particles. These authors stated that, because of the small difference in sedimentation rate between mitochondria and lysosomes, a delicate procedure was required to obtain partial separation of these two types of particle. A separation in good yield of the three types of particle in the adrenal medulla by differential centrifugation in 0·3 M sucrose would therefore be even more difficult.

A clear separation of the particles containing the lysosomal enzymes from both chromaffin granules and mitochondria was made possible by the use of a sucrose density gradient. Separation of chromaffin granules from mitochondria on a sucrose gradient was first achieved by Blaschko *et al.* (1957). It has now been found that most of the lysosomes were present in a distinct band that corresponded to an initial sucrose concentration of 1·6 M. Rat liver lysosomes had a similar median equilibrium density in sucrose in the very precise experiments of Beaufay *et al.* (1964). The same authors found that acid phosphatase had a slightly different distribution from that of deoxyribonuclease in a sucrose gradient. Figure 3 shows that the same is true of these enzymes in the adrenal medulla. A bimodal distribution of lysosomal enzymes in the sucrose gradient was also found by Beaufay *et al.* (1964).

Four enzymes have been considered to be present in chromaffin granules as a result of their distribution in fractions obtained by centrifugation: acid phosphatase (Hillarp & Falck, 1956), ribonuclease (Philippu & Schümann, 1964), dopamine-β-hydroxylase (Kirshner, 1959) and adenosinetriphosphatase (see Banks, 1965). The present work makes it very likely that acid phosphatase and ribonuclease are in fact present in lysosomes. The sucrose gradient used in our experiments gives a highly purified chromaffin granule fraction. Fractions 6 and 7 contained 51% of the catecholamines present in the large-granule fraction, but less than 5% of each acid hydrolase.

Coupland (1965) has described lysosome-like structures in electron micrographs of rat adrenal medulla. He also observed an increased vacuolation of lysosomes after stimulation of the gland. It would be interesting to know whether this observation indicates an involvement of lysosomes in the release of catecholamines. A role of lysosomes in the secretion of thyroid hormones has been proposed on the basis of histochemical studies on the distribution of lysosomes and 'colloid droplets' after stimulation of the thyroid gland (Novikoff, 1963; Novikoff, Essner & Quintana, 1964; Wollman, Spicer & Burnstone, 1964). It has also been

reported that the secretion of thyroid stimulating hormone from the adenohypophysis is accompanied by an activation of acid phosphatase (Schreiber, 1961; Lojda & Schreiber, 1964). There is no direct evidence in the adrenal medulla for an interaction between lysosomal enzymes and chromaffin granules, but it is interesting that ribonuclease caused the release of catecholamines from chromaffin granules *in vitro* (Philippu & Schümann, 1963).

The probable role of lysosomes in the autolysis of tissues following cell death has been discussed by several authors (e.g. de Duve, 1959, and Novikoff, 1961). It is well known to anatomists that the adrenal medulla rapidly undergoes autolysis after death, and this may be a result of its relatively high content of lysosomal enzymes. The adrenal glands were called 'capsulae atrabilariae' by Caspar Bartholinus the Elder (1611) because the gland appeared to him as a capsule enclosing a cavity that contained a black fluid. The cavity was probably due to autolysis of chromaffin tissue *post mortem*.

We are very grateful to Dr H. Blaschko for his advice and for his interest in this work. One of us (A.D.S.) is the holder of an M.R.C. scholarship and the other (H.W.) is a Linacre House Postgraduate Student.

This work has been supported by the Medical Research Council and by the U.S. Air Force Office of Aerospace Research (Grant AF EOAR 64–12).

REFERENCES

ANSON, M. L. (1938). The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. gen. Physiol.* **22**, 79–89.

BANKS, P. (1965). The adenosine-triphosphatase activity of adrenal chromaffin granules. *Biochem. J.* **95**, 490–496.

BARTHOLINUS, C. (1611). *Anatomicae institutiones corporis humani*, p. 114. Wittenberg: Raab.

BAUDHUIN, P., BEAUFAY, H., RAHMAN-LI, Y., SELLINGER, O. Z., WATTIAUX, R., JACQUES, P. & DE DUVE, C. (1964). Tissue fractionation studies 17. Intracellular distribution of monoamineoxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat liver tissue. *Biochem. J.* **92**, 179–184.

BEAUFAY, H., BENDALL, D. S., BAUDHUIN, P., WATTIAUX, R. & DE DUVE, C. (1959). Tissue fractionation studies 13. Analysis of mitochondrial fractions from rat liver by density-gradient centrifuging. *Biochem. J.* **73**, 628–637.

BEAUFAY, H., JACQUES, P., BAUDHUIN, P., SELLINGER, O. Z., BERTHET, J. & DE DUVE, C. (1964). Tissue fractionation studies 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem. J.* **92**, 184–205.

BLASCHKO, H., BORN, G. V. R., D'IORIO, A. & EADE, N. R. (1956). Observations on the distribution of catecholamines and adenosinetriphosphate in the bovine adrenal medulla. *J. Physiol.* **133**, 548–557.

BLASCHKO, H., HAGEN, J. M. & HAGEN, P. (1957). Mitochondrial enzymes and chromaffin granules. *J. Physiol.* **139**, 316–322.

BLASCHKO, H., HAGEN, P. & WELCH, A. D. (1955). Observations on the intracellular granules of the adrenal medulla. *J. Physiol.* **129**, 27–49.

COUPLAND, R. E. (1965). Electron microscopic observations on the structure of the rat adrenal medulla. 1. The ultrastructure and organization of chromaffin cells in the normal adrenal medulla. *J. Anat., Lond.*, **99**, 231–254.

DE DUVE, C. (1959). Lysosomes, a new group of cytoplasmic particles. In *Subcellular Particles*, ed. HAYASHI, T. New York: Ronald Press Co.

DE DUVE, C., PRESSMAN, B. C., GIANETTO, R., WATTIAUX, R. & APPELMANS, F. (1955). Tissue fractionation studies 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**, 604–617.

ERÄNKÖ, O. (1952). On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. *Acta anat.* **15**, suppl. 17, 1–60.

VON EULER, U. S. & HAMBERG, U. (1949). Colorimetric determination of noradrenaline and adrenaline. *Acta physiol. scand.* **19**, 74–84.

GIANETTO, R. & DE DUVE, C. (1955). Tissue fractionation studies 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. *Biochem. J.* **59**, 433–438.

HILLARP, N.-Å. (1958). Isolation and some biochemical properties of the catecholamine granules in the cow adrenal medulla. *Acta physiol. scand.* **43**, 82–96.

HILLARP, N.-Å. & FALCK, B. (1956). Localization of acid phosphatase in the adrenal medullary cell. *Acta endocr., Copenh.*, **22**, 95–106.

KIRSHNER, N. (1959). Biosynthesis of adrenaline and noradrenaline. *Pharmac. Rev.* **11**, 350–357.

KOENIG, H., MYLROIE, R., GAINES, D., GRAY, R. & MCDONALD, T. (1965). Studies of catecholamine storage particles. *Neurology*, **15**, 289.

LOJDA, Z. & SCHREIBER, V. (1964). Histochemical demonstration of the inhibition of rat adenohypophyseal acid phosphatase by valine^3-oxytocin. *J. Histochem. Cytochem.* **12**, 855–856.

MALMSTROM, G. & GLICK, D. (1952). Studies in histochemistry 25. Determination of phenolsulphatase in microgram quantities of tissue and its distribution in the adrenal of several species. *Arch. Biochem. Biophys.* **40**, 56–67.

MCILVAINE, T. C. (1921). A buffer solution for colorimetric comparison. *J. biol. Chem.* **49**, 183–186.

NOVIKOFF, A. B. (1961). Lysosomes and related particles. In *The Cell*, vol. 2, ed. BRACHET J. & MIRSKY, A. E. London, New York: Academic Press.

NOVIKOFF, A. B. (1963). Lysosomes in thyroid epithelium of untreated, TSH-stimulated and I^{131}-irradiated rats. *Biol. Bull. mar. biol. Lab., Woods Hole*, **125**, 358–359.

NOVIKOFF, A. B., ESSNER, E. & QUINTANA, N. (1964). Golgi apparatus and lysosomes. *Fedn Proc.* **23**, 1010–1022.

PHILIPPU, A. & SCHÜMANN, H. J. (1963). Effect of ribonuclease on the ribonucleic acid, adenosine triphosphate and catecholamine content of medullary granules. *Nature, Lond.*, **198**, 795–796.

PHILIPPU, A. & SCHÜMANN, H. J. (1964). Ribonucleaseaktivität isolierter Nebennierenmarkgranula. *Experientia*, **20**, 547–548.

RACKER, E. (1950). Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. *Biochim. biophys. Acta*, **4**, 211–214.

ROTH, J. S. & MILSTEIN, S. W. (1952). Ribonuclease 1. A new assay method with P^{32}-labelled yeast ribonucleic acid. *J. biol. Chem.* **196**, 489–498.

ROY, A. B. (1956). The sulphatase of ox liver. 5. Sulphatase C. *Biochem. J.* **64**, 651–657.

ROY, A. B. (1958). Comparative studies on liver sulphatases. *Biochem. J.* **68**, 519–528.

SCHNEIDER, W. C. & HOGEBOOM, G. H. (1952). Intracellular distribution of enzymes 9. Certain purine-metabolizing enzymes. *J. biol. Chem.* **195**, 161–166.

SCHREIBER, V. (1961). A hypothalamic factor activating pituitary acid phosphatases and the secretion of TSH. *Acta Univ. Carol. Med.* **7**, 33–87.

SMITH, A. D. & WINKLER, H. (1965). Acid nucleases of the bovine adrenal medulla. *Nature, Lond.*, **207**, 634.

TODD, P. E. E. & TRIKOJUS, V. M. (1960). Purification and properties of adrenal acid proteinase. *Biochim. biophys. Acta*, **45**, 234–242.

WETZSTEIN, R. (1957). Electronenmikroskopische Untersuchungen am Nebennierenmark von Maus, Meerschweinchen und Katze. *Z. Zellforsch. mikrosk. Anat.* **46**, 517–576.

WOLLMAN, S. H., SPICER, S. S. & BURNSTONE, M. S. (1964). Localization of esterase and acid phosphatase in granules and colloid droplets in rat thyroid epithelium. *J. cell Biol.* **21**, 191–202.