J. Physiol. (1969), **205**, *pp.* 103–114
With 4 text-figures
Printed in Great Britain

BOVINE SPLENIC NERVE: CHARACTERIZATION OF NORADRENALINE-CONTAINING VESICLES AND OTHER CELL ORGANELLES BY DENSITY GRADIENT CENTRIFUGATION

BY H. HÖRTNAGL, HEIDE HÖRTNAGL AND H. WINKLER

From the Department of Pharmacology, University of Innsbruck, Austria

(*Received* 14 *April* 1969)

SUMMARY

1. Homogenates of bovine splenic nerves were subjected to differential and sucrose density gradient centrifugation. From the low-speed supernatant a high-speed sediment (mitochondria, lysosomes, microsomes and noradrenaline (NA) vesicles) was obtained. By density gradient centrifugation of this sediment it was shown that NA vesicles are slightly less dense than mitochondria, but denser than microsomes.

2. In further experiments a mitochondrial and a microsomal sediment were obtained. The mitochondrial sediment was fractionated with a short centrifugation time over a density gradient ranging from 0·6 to 1·2 M sucrose. Mitochondria (fumarase and succinate-dehydrogenase) and lysosomes (acid ribonuclease and deoxyribonuclease) sedimented to the bottom of the tube. The highest concentration of NA vesicles was found in a medium position. There was only a small amount of microsomes (glucose-6-phosphatase) present.

3. The microsomal sediment was centrifuged for 150 min over a density gradient ranging from 0·8 to 1·4 M sucrose. The microsomes remained on the top of the gradient. There were also some mitochondria and lysosomes present. The NA vesicles were found in highest concentration in the middle of the gradient (at about 1·2 M sucrose).

4. With the use of these two density gradients, the subcellular distribution of dopamine-β-hydroxylase, monoamine oxidase and ATPase was studied. Dopamine-β-hydroxylase was found to be localized in the NA vesicles. Monoamine oxidase was mainly recovered in mitochondria; a small part of the enzyme appeared to be microsomal. ATPase was present in microsomal elements.

INTRODUCTION

Catecholamines are stored in subcellular organelles both in sympathetic nerves and in the adrenal medulla. The storage organelles of the latter organ, i.e. the chromaffin granules, have been separated from other cell organelles by differential and density gradient centrifugation (see review by Smith, 1968). This achievement was based on a thorough knowledge of the behaviour of all the cell particles, and not only of the chromaffin granules, during the centrifugation steps. Such knowledge was obtained by the determination of enzymes and constituents which are characteristic of the different cell particles, i.e. chromaffin granules, mitochondria (Blaschko, Hagen & Hagen, 1957), lysosomes (Smith & Winkler, 1966) and microsomes (Smith & Winkler, 1968; Winkler, 1969).

The present study is an attempt to obtain similar information for the cell organelles present in homogenates of bovine splenic nerve. It will be shown that noradrenaline-containing vesicles (NA vesicles) can be differentiated from microsomes, mitochondria and lysosomes. Based on these results evidence for the localization of dopamine-β-hydroxylase in NA vesicles will be provided. In addition, the subcellular localization of monoamine oxidase and ATPase will be reported.

METHODS

Subcellular fractionation. Bovine splenic nerves were obtained in the slaughterhouse and immediately put on ice. After the removal of the connective tissue, the nerves (7–14 g) were chopped with a knife. The mince was suspended in 0·3 M sucrose to give a 1:8 (w/v) suspension. Homogenization was performed with a Potter–Elvehjem homogenizer (radial clearance: 0·08 cm) by moving the Teflon pestle up and down ten times while rotating it at 475 rev/min. The homogenate was centrifuged for 20 min at 800 *g* (all *g* values are maximum). The sediment of unbroken cells and nuclei was resuspended once with 30 ml. 0·3 M sucrose and centrifuged again. The combined supernatants were filtered through a double layer of gauze and then centrifuged for 20 min at 12000 *g* (MSE ultracentrifuge, 8 × 25 ml. angle head) in order to obtain a mitochondrial sediment. The supernatant was centrifuged for 60 min at 135,000 *g* which gave a microsomal sediment. These sediments were resuspended with a pipette in 1·7 ml. 0·3 M sucrose. In some preliminary experiments the low speed supernatant was centrifuged for 60 min at 135,000 *g* in order to sediment mitochondria and microsomes together.

Density gradients were prepared by pipetting sucrose solutions of decreasing molarity above each other in a centrifuge tube (5 ml. tubes of the 3 × 5 ml. swing out head, MSE ultracentrifuge). First 0·25 ml. of 2·0 M sucrose was pipetted into the tubes and then 7 × 0·5 ml. of a series of sucrose solutions, the concentration of which decreased in steps of 0·1 M. The molarities used are given in the Results section. After preparation, the gradients were left in the cold (2° C) for at least 7 hr. Before centrifugation, 0·5 ml. of the resuspended sediments was layered on top of the gradients. Centrifugation times are given in the Results section. After centrifugation the bottom of each tube was pierced with a needle and up to eight fractions were collected. The

fractions from three gradient tubes were pooled. The pooled fractions were diluted with an equal volume of Tris/Na succinate buffer (0·005 M, pH 5·9). Half of each diluted fraction was used for the NA determination. The proteins were precipitated with perchloric acid (final concentration: 3 %, with 0·25 % ethylenediaminetetraacetic acid (EDTA) and 0·1 % ascorbic acid). Catecholamines were determined in the supernatant as described by Euler & Lishajko (1959). The results given were not corrected for the actual recovery of NA.

Enzyme assays. Dopamine-β-hydroxylase was measured in a final volume of 1·0 ml. according to Friedman & Kaufman (1965). The incubation mixture contained the following components in mM concentrations: potassium phosphate buffer (pH 4·5–7·5) 100, fumaric acid 25, ATP 5, ascorbic acid 1, tranylcypromine sulphate 0·5, [U-^{3}H]tyramine (specific activity 2·1 c/μ-mole) 0·00076 and catalase (3500 Sigma units). Up to 0·3 ml. tissue samples was added. The mixture was incubated for 30 min at 37° C. The determination of the octopamine formed was performed exactly as described by Friedman & Kaufman (1965). Blanks containing water instead of tissue samples were carried through the entire procedure. The solubility of dopamine-β-hydroxylase in water was determined with a fraction of the microsomal gradient containing a high enzyme concentration. To 0·6 ml. of the fraction 0·1 ml. Tris/Na succinate (0·005 M, pH 5·9) was added and the suspension was then centrifuged at 70,000 *g* for 15 min. The sediment was extracted once with 0·2 ml. Tris/Na succinate buffer (0·005 M), re-sedimented and then extracted once with Tris/Na succinate buffer with 0·2 M-NaCl added. Between the extraction steps the samples were frozen and thawed. The combined supernatants were finally spun at 160,000 *g* for 60 min. Dopamine-β-hydroxylase was then determined in the combined supernatants and in the final sediment.

Monoamine oxidase was determined according to Wurtman & Axelrod (1963). The incubation mixture consisted of 0·1 ml. Na borate buffer (pH 6·5–10, 0·25 M), up to 0·3 ml. tissue samples and 0·1 ml. substrate in water (0·05 μ-mole [^{14}C]2-tryptamine bisuccinate, specific activity 10·9 mc/m-mole). The mixture was incubated for 20 min at 37° C. Blanks containing water instead of tissue samples were incubated together with the experimental tubes. After incubation 0·8 ml. 2 N-HCl was added, followed by 6 ml. toluene to extract the deaminated radioactive material. After shaking and centrifuging, 4 ml. of the toluene layer was transferred to scintillation vials containing 10 ml. toluene with 4 g 2·5-diphenyloxazole and 100 mg (1·4-bis-[2(5-phenyloxazolyl)]-benzene per litre.

Estimated were: malate hydrolyase (fumarase; Racker, 1950), acid ribonuclease and deoxyribonuclease (Smith & Winkler, 1966), glucose-6-phosphatase (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), Mg^{2+}-activated ATPase (Kirshner, Kirshner & Kamin, 1966) and succinate-dehydrogenase (Porteous & Clark, 1965). Protein was estimated by the micro-biuret method.

Materials. Tranylcypromine sulphate was kindly donated by Smith, Kline and French (Philadelphia). Catalase (crystalline from beef liver) and ATP (disodium) was purchased from Sigma (London); yeast ribonucleic acid, calf thymus deoxyribonucleic acid and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)5-phenyltetrazolium chloride from British Drug Houses Ltd. [^{14}C]2-tryptamine bisuccinate and [U-^{3}H]*p*-hydroxyphenylethylamine (tyramine) were obtained from NEN Chemicals GmbH.

RESULTS

Differential and density gradient centrifugation. Homogenates of bovine splenic nerve contained 104 ± 6 (S.D., $n = 3$) ng NA/mg protein. By differential centrifugation of the homogenates it was found that $48{\cdot}8 \pm 16$ %

(n = 8) of the total recovered NA was present in the low speed sediment, 17·4 ± 4% (n = 8) in the high speed sediment (mitochondria and microsomes) and 33·8 ± 16·3% (n = 8) in the final supernatant. Microsomal pellets contained about three times more NA than did the mitochondrial pellets.

In a first series of experiments the high speed sediment was subjected to density gradient centrifugation. In a density gradient ranging from 0·6 to 1·2 M sucrose (centrifuged for 45 min at 127,000 g) NA vesicles sedimented to a central position. Microsomes characterized by glucose-6-phosphatase remained in the top layer; mitochondria, marked by fumarase, sedimented to the bottom of the tube. In various gradients and even after prolonged centrifugation mitochondria always appeared slightly denser than NA vesicles. Microsomes were always less dense.

In order to obtain a better differentiation of the various particles a mitochondrial and microsomal sediment were used separately. The mitochondrial sediment was centrifuged for 45 min over a gradient ranging from 0·6 to 1·2 M sucrose. After centrifugation a dark brown layer was visible at the bottom of the tube. There was some opacity above this layer but no distinct regions could be seen. The microsomal sediment was centrifuged for 150 min over a gradient ranging from 0·8 to 1·4 M sucrose. After centrifugation a dark brown layer was present in the top fraction of the gradient. Below the layer the opacity decreased continually from the top to the bottom of the tube. The results of the analyses of the fractions from the gradients are shown in Figs. 1 and 2, where, in each histogram, the columns from left to right represent fractions from the top to the bottom of the centrifuge tube. Since the arbitrary units in both Figures are the same, a comparison of the distribution patterns gives qualitative and quantitative information.

Mitochondria were characterized by two enzymes, namely fumarase and succinate dehydrogenase. In the mitochondrial gradient the greater part of the mitochondria sediments to the bottom of the tube (Fig. 1). The microsomal sediment is contaminated by quite a few mitochondria which equilibrate throughout the gradient with a slight preference for the top fractions (see Fig. 2). Succinate-dehydrogenase, which is an insoluble enzyme, appears to be a better 'marker' enzyme than the soluble fumarase which exhibits a significant peak of activity in the top fractions of the gradients. This is probably due to extramitochondrial fumarase, which is also found in large amounts in the final supernatant.

Acid ribonuclease and deoxyribonuclease are typical lysosomal enzymes (De Duve & Wattiaux, 1966). The larger part of these enzymes is found in the mitochondrial sediment (compare Figs. 1 and 2) and they have a distribution similar to that of mitochondria in the gradient (see Fig. 1). The

peak of enzyme activity present in the top fractions of both gradients (see Figs. 1 and 2) is probably due to enzyme not contained in particles. In the microsomal gradient, in which the sucrose molarity rises to 1·4 M, acid ribonuclease sediments into slightly denser sucrose than do the mitochondrial enzymes. It is already known, e.g. from the adrenal medulla (Smith & Winkler, 1966; Laduron & Belpaire, 1968), that lysosomes are denser than mitochondria.

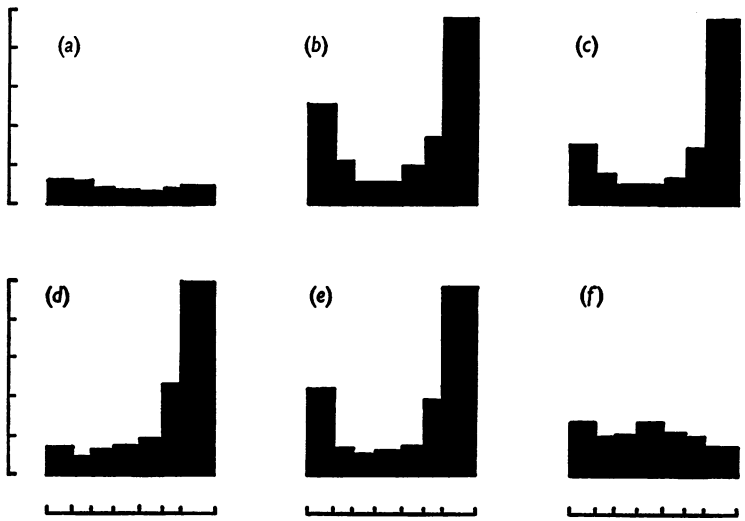

Fig. 1. Analysis of fractions from the sucrose gradient centrifugation of the mitochondrial sediment. The gradient ranged from 0·6 to 1·2 M sucrose and was centrifuged at 127,000 *g* for 45 min. The columns from left to right in each histogram correspond to the fractions from the top to the bottom in the centrifuge tube. The abscissa is divided according to the volumes of the fractions. The ordinates are arbitrary units/ml. and the actual value of 1 arbitrary unit is shown in parentheses below. The values for the enzymes are expressed in μ-mole substrate utilized/hr/ml. fraction. (*a*) Glucose-6-phosphatase (1·2); (*b*) acid deoxyribonuclease (0·065); (*c*) acid ribonuclease (0·6); (*d*) succinate-dehydrogenase (0·6); (*e*) fumarase (1·6); (*f*) NA (125 ng/ml.). The values for the distribution pattern of NA are the means of four experiments and those of ribonuclease of two experiments. Recoveries ranged from 83 to 112 %.

Glucose-6-phosphatase is considered to be a good marker enzyme for microsomal elements in several tissue (see Reid, 1967) including the adrenal medulla (Smith & Winkler, 1968; Winkler, 1969). In the subcellular fractions of the splenic nerve it was mainly found in the microsomal pellet (compare Figs. 1 and 2); on the microsomal gradient it equilibrated in sucrose of low molarity, which is consistent with the presence of this enzyme in microsomal elements. The highest concentration of protein was also found in the top fractions of the gradient (see Fig. 2).

In both gradients (see Figs. 1 and 2) a relatively high concentration of NA was recovered from the top fractions, which corresponded to the re-suspended sediments layered on top of the gradients before centrifugation. It can be assumed that some of the NA found in these fractions represents unbound amine since there is always some leakage of NA from the vesicles when the particulate sediments are re-suspended by pipetting. In addition, in bovine splenic nerve there is no evidence for the presence of a distinct light NA-vesicle fraction which might not have entered the gradients used

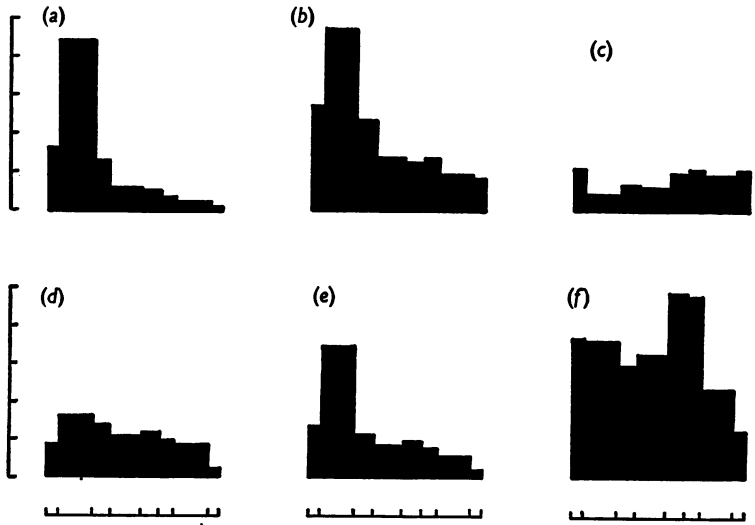

Fig. 2. Analysis of fractions from the sucrose gradient centrifugation of the microsomal sediment. The gradient ranged from 0·8 to 1·4 M sucrose and was centrifuged at 127,000 *g* for 150 min. The arbitrary units are the same as in Fig. 1 (in addition, protein 0·77 mg/ml.). In the fraction with the highest NA content there were 501 ± 141 ($n = 5$) ng NA (free base)/mg protein. (*a*) Glucose-6-phosphatase; (*b*) protein; (*c*) acid ribonuclease; (*d*) succinate dehydrogenase; (*e*) fumarase; (*f*) NA. The values for the distribution pattern of NA are the means of five experiments, those of protein of four and those of glucose-6-phosphatase of three experiments. Recoveries ranged from 76 to 107 %.

in the present study (Roth, Stjärne, Bloom & Giarman, 1968). The vesicles containing the NA were distributed throughout the mitochondrial gradient, with the highest concentration in a middle fraction (corresponding to 0·9 M sucrose). In the microsomal gradient (see Fig. 2) the NA vesicles were concentrated in a layer of the gradient corresponding to 1·2 M sucrose.

Subcellular localization of dopamine-β-hydroxylase, monoamine oxidase (MAO) and ATPase. Activity of dopamine-β-hydroxylase in homogenates of bovine splenic nerve could be demonstrated only in the presence of sodium *p*-chloromercuribenzoate. The optimal concentration of

this substance was 0·1 mM. The activity of the enzyme at different pH is shown in Fig. 3; there is an optimum at pH 6·5.

Since the break-down of substrate by the gradient fractions was proportional to enzyme concentration, a quantitative distribution of dopamine-β-hydroxylase in the density gradients could be obtained. This is shown in Fig. 4*a*. A comparison with Figs. 1 and 2 reveals that the distribution of dopamine-β-hydroxylase matches quantitatively and qualitatively that of the particulate NA and not that of mitochondrial, lysosomal

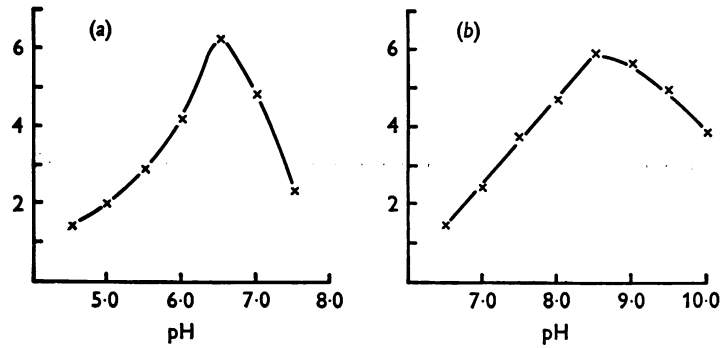

Fig. 3. Effect of pH on dopamine-β-hydroxylase (*a*) and MAO (*b*) activity. Dopamine-β-hydroxylase was determined in K phosphate buffers (0·1 M) and MAO in Na borate buffers (0·25 M). For the dopamine-β-hydroxylase assay a fraction from the microsomal gradient with high enzyme activity, for the MAO assay the fraction from the bottom of the mitochondrial gradient were used. One unit of the ordinate corresponds to p-mole substrate for (*a*) and to n-mole substrate for (*b*).

or microsomal enzymes. In the particular experiments illustrated in Fig. 4*a* the NA content of the various fractions was also estimated. Therefore, ratios of the activity of dopamine-β-hydroxylase (in p-mole substrate utilized/hr) to NA (μg free base) could be obtained. In the two upper fractions of both gradients, where non-particulate NA is found, there is relatively little activity of the insoluble dopamine-β-hydroxylase and therefore the enzyme/NA ratios were found to be low, ranging from 78 to 182. In the other fractions from both gradients the average value of these ratios was 291 ± 36 ($n = 11$). The constancy of the ratios in these fractions is a good indication for the presence of NA and dopamine-β-hydroxylase within the same particle. The insolubility of dopamine-β-hydroxylase is demonstrated by the fact that only 8 % of the enzyme activity present in the fractions from the density gradient could be solubilized by extraction with buffer.

MAO activity was measured at the optimal pH of 8·5 (see Fig. 3). The enzymic activity was completely destroyed by boiling for 10 min and was

completely inhibited by 2·0 mM tranylcypromine. The break-down of substrate was proportional to the amount of enzyme. The distribution of MAO in the mitochondrial gradient (see Fig. 4) is consistent with the presence of this enzyme in mitochondria. In the microsomal gradient the activity of MAO found in the fraction where the NA vesicles are concentrated can be attributed to the contaminatiing mitochondria, since the

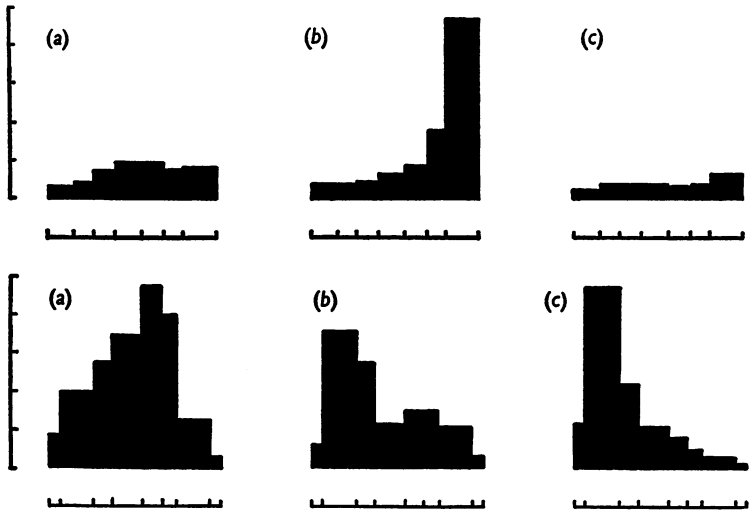

Fig. 4. Distribution patterns of dopamine-β-hydroxylase, MAO and ATPase in the mitochondrial (upper diagrams) and microsomal gradients (lower diagrams).

The values of the arbitrary units of the ordinate (defined as in Fig. 1) are given in parentheses below. (*a*) Dopamine-β-hydroxylase ($0{\cdot}041 \times 10^{-3}$), (*b*) MAO (0·94), (*c*) ATPase (4·3). Recoveries ranged from 90 to 97 %.

mitochondrial enzyme succinate dehydrogenase exhibits a similar concentration in these fractions. However, this enzyme has no marked peak of activity in the top fraction (see Fig. 2) where a high concentration of MAO activity is found.

Most of the Mg^{2+} ATPase (measured at pH 7·4) appears to be localized in microsomal elements because this enzyme shows a distribution pattern (see Fig. 4) similar to that of glucose-6-phosphatase in both gradients (see Figs. 1 and 2).

DISCUSSION

When homogenates of bovine splenic nerves were subjected to differential centrifugation, the major part of the NA was found in the low speed sediment and in the final supernatant; in the high speed sediment which was used as the starting material for the characterization of the NA vesicles only 20 % of the NA were present. Similar figures, i.e. a relatively

small recovery of NA in the high speed sediment, have already been reported for bovine splenic nerve (Euler & Hillarp, 1956; Schümann, 1958; Euler & Lishajko, 1961; Schümann, Schmidt & Philippu, 1966), for heart tissue (Wegmann & Kako, 1961; Potter & Axelrod, 1963; Campos, Stitzel & Shideman, 1963; Iversen & Whitby, 1963; Schümann, Schnell & Philippu, 1964; Glassman, Angelakos & McNary, 1965; Euler & Lishajko, 1965; Taylor, Chidsey, Richardson, Cooper & Michaelson, 1966), for stellate ganglia (Schümann *et al.* 1966) and for smooth muscle tissue (Austin, Chubb & Livett, 1967). In the adrenal medulla, on the other hand, the major part of the hormones is found in the large granule fraction from which the chromaffin granules can be isolated (see Smith, 1968).

By density gradient centrifugation the NA vesicles could be differentiated from mitochondria, microsomes and lysosomes, which were characterized by typical 'marker' enzymes. The highest concentration of NA vesicles in the mitochondrial gradient was found in a layer corresponding to 0·9 M sucrose. Since these gradients were centrifuged for a short time, the presence of a peak of NA vesicles in sucrose of this low molarity probably reflects a slow sedimentation rate rather than a low density of these particles. In the microsomal gradient which was centrifuged much longer NA vesicles were concentrated in a layer corresponding to 1·2 M sucrose. Such a relatively high density of the NA vesicles of bovine splenic nerve has already been described (Roth *et al.* 1968; Burger, Philippu & Schümann, 1969). In the rat heart, NA-containing vesicles were reported to have a very low density comparable to that of microsomes (see Potter, 1966, 1967). However, the recent work of Roth *et al.* (1968) demonstrates that in this tissue also, a clearly defined population of relatively dense NA vesicles exists.

Activity of dopamine-β-hydroxylase in homogenates of bovine splenic nerve could be demonstrated only in the presence of sodium *p*-chloromercuribenzoate. This is probably due to the presence of endogenous inhibitors such as have been demonstrated in the adrenal medulla and other tissues (Duch, Viveros & Kirshner, 1968; Viveros, Arqueros & Kirshner, 1968; Austin, Livett & Chubb, 1967; Nagatsu, Kuzuya & Hidaka, 1967). Viveros *et al.* (1968) have shown that the inhibitors in the adrenal medulla are inactivated by *p*-mercuribenzoate. In previous studies it has been reported that 'highly purified' NA vesicles contain dopamine-β-hydroxylase; however, no quantitative distribution of this enzymes over the various fractions obtained by differential and density gradient centrifugation has been given (Potter, 1966; 1967; Stjärne, 1966). With the present methods more direct evidence for the localization of dopamine-β-hydroxylase in NA vesicles has been obtained. This enzyme was distributed in the fractions from the density gradient in the same way as the particle-bound NA and we can therefore conclude

that dopamine-β-hydroxylase is localized in NA vesicles. A similar result was independently obtained for NA vesicles from ox splenic nerve by De Potter, Smith & de Schaepdryver (1969).

ATPase (Mg^{2+} activated) was distributed in the density gradients in the same way as glucose-6-phosphatase, which suggests a microsomal localization of this enzyme. However, in careful experiments with fractions from density gradients, Burger *et al.* (1969) demonstrated that ATPase in a fraction rich in NA was relatively less activated by Ca^{2+} compared with a fraction corresponding to the microsomes. This difference was attributed to special properties of an ATPase in NA vesicles. In any case, the present results indicate quite clearly that the bulk of ATPase activity must be present in microsomes and that NA vesicles can only contain a relatively small amount.

MAO activity was found by Roth & Stjärne (1966) in a subcellular fraction from the splenic nerve containing the NA vesicles. The possibility was considered that the NA vesicles actually contain some of this enzyme. A similar suggestion was made by De Champlain, Axelrod, Krakoff & Müeller (1968) for MAO activity in the salivary gland. On the other hand, Stjärne, Roth & Giarman (1968) discuss the possibility that the MAO activity found in the fraction containing the NA vesicles is due to microsomal elements, but a differentiation of microsomes and NA vesicles was not presented. NA vesicles of vas deferens can at least be partly separated from MAO activity by density gradient centrifugation which is good evidence against a localization of MAO in NA vesicles (Jarrot & Iversen, 1968). The present study has shown that NA vesicles from bovine splenic nerve contain little, if any, activity of MAO. Most of this enzyme is found in the mitochondrial fraction. In the microsomal gradients some of the activity can be attributed to contaminating mitochondria, but the peak of MAO activity in the top fraction was not shown by the mitochondrial enzyme succinate-dehydrogenase, which may be taken as an indication for a microsomal localization of this enzyme (compare Jarrot & Iversen, 1968).

It was the purpose of the present study not only to characterize the sedimentation properties of NA vesicles but also to provide information about those of the other cell particles. Evidence for the subcellular localization of dopamine-β-hydroxylase, ATPase and MAO has been presented. In addition, any claim that NA vesicles have been obtained in a highly purified form can now be assessed by determining the degree of contamination with the 'marker' enzymes described here.

We are grateful to Dr A. D. Smith (Oxford) for his help in preparing this manuscript and to Miss Ch. Kranewitter for excellent technical assistance.

REFERENCES

AUSTIN, L., CHUBB, I. W. & LIVETT, B. G. (1967). The subcellular localization of catecholamines in nerve terminals in smooth muscle tissue. *J. Neurochem.* **14**, 473–478.

AUSTIN, L., LIVETT, B. G. & CHUBB, I. W. (1967). Biosynthesis of noradrenaline in sympathetic nervous tissue. *Circulation Res.* XX, suppl. III, 111–117.

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

BURGER, A., PHILIPPU, A. & SCHÜMANN, H. J. (1969). ATP-Spaltung und Aminaufnahme durch Milznervengranula. *Arch. exp. Path. Pharmak.* **262**, 208–220.

CAMPOS, H. A., STITZEL, R. E. & SHIDEMAN, F. E. (1963). Actions of tyramine and cocaine on catecholamine levels in subcellular fractions of the isolated cat heart. *J. Pharmac. exp. Ther.* **141**, 290–300.

DE CHAMPLAIN, J., AXELROD, J., KRAKOFF, L. R. & MUELLER, R. A. (1968). Microsomal localization of monoamine oxidase in the heart and salivary gland. *Fedn Proc.* **27**, 399.

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

DE DUVE, C. & WATTIAUX, R. (1966). Functions of lysosomes. *A. Rev. Physiol.* **28**, 461–492.

DE POTTER, W. P., SMITH, A. D. & DE SCHAEPDRYVER, A. F. (1969). Subcellular fractionation of bovine splenic nerves. *Archs int. Pharmacodyn. Thér.* (In the Press.)

DUCH, D. S., VIVEROS, O. H. & KIRSHNER, N. (1968). Endogenous inhibitors in adrenal medulla of dopamine-β-hydroxylase. *Biochem. Pharmac.* **17**, 255–264.

EULER, U. S. v. & HILLARP, N. A. (1956). Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. *Nature, Lond.* **177**, 44.

EULER, U. S. v. & LISHAJKO, F. (1959). The estimation of catecholamines in urine. *Acta physiol. scand.* **45**, 122–132.

EULER, U. S. v. & LISHAJKO, F. (1961). Noradrenaline release from isolated nerve granules. *Acta physiol. scand.* **51**, 193–203.

EULER, U. S. v. & LISHAJKO, F. (1965). Free and bound noradrenaline in the rabbit heart. *Nature, Lond.* **205**, 179.

FRIEDMAN, S. & KAUFMAN, S. (1965). 3,4-Dihydroxyphenylethylamine β-hydroxylase: Physical properties, copper content and role of copper in the catalytic activity. *J. biol. Chem.* **240**, 4763–4773.

GLASSMAN, P. M., ANGELAKOS, E. T. & MCNARY, W. F. (1965). Catecholamine-containing fractions of dog heart homogenates. *Life Sci. Oxford* **4**, 1727–1734.

IVERSEN, L. L. & WHITBY, L. G. (1963). The subcellular distribution of catecholamines in normal and tyramine-depleted mouse hearts. *Biochem. Pharmac.* **12**, 582–584.

JARROT, B. & IVERSEN, L. L. (1968). Subcellular distribution of monoamine oxidase activity in rat liver and vas deferens. *Biochem. Pharmac.* **17**, 1619–1625.

KIRSHNER, N., KIRSHNER, A. G. & KAMIN, D. L. (1966). Adenosine triphosphatase activity of adrenal medulla catecholamine granules. *Biochim. biophys. Acta* **113**, 332–335.

LADURON, P. & BELPAIRE, F. (1968). Tissue fractionation and catecholamines-II. Intracellular distribution patterns of tyrosine hydroxylase, dopa decarboxylase, dopamine-β-hydroxylase, phenylethanolamine *N*-methyltransferase and monoamine oxidase in adrenal medulla. *Biochem. Pharmac.* **17**, 1127–1140.

NAGATSU, T., KUZUYA, H. & HIDAKA, H. (1967). Inhibition of dopamine-β-hydroxylase by sulfhydryl components and the nature of the natural inhibitors. *Biochim. biophys. Acta* **139**, 319–327.

PORTEOUS, J. W. & CLARK, B. (1965). The isolation and characterization of subcellular components of the epithelial cells of rabbit small intestine. *Biochem. J.* **96**, 159–171.

POTTER, L. T. (1966). Storage of norepinephrine in sympathetic nerves. *Pharmac. Rev.* **18**, 439–451.

POTTER, L. T. (1967). Role of intraneuronal vesicles in the synthesis, storage and release of noradrenaline. *Circulation Res.* XX, suppl. III, 13–24.

POTTER, L. T. & AXELROD, J. (1963). Subcellular localization of catecholamines in tissues of the rat. *J. Pharmac. exp. Ther.* **142**, 291–298.

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

REID, E. (1967). Membrane systems. *Enzyme Cytology*, ed. RODYN, D. B., pp. 321–406. London: Academic Press.

ROTH, R. H. & STJÄRNE, L. (1966). Monoamine oxidase activity in the bovine splenic nerve granule preparation. *Acta physiol. scand.* **68**, 342–346.

ROTH, R. H., STJÄRNE, L., BLOOM, F. E. & GIARMAN, N. J. (1968). Light and heavy norepinephrine storage particles in the rat heart and in bovine splenic nerve. *J. Pharmac. exp. Ther.* **162**, 203–212.

SCHÜMANN, H. J. (1958). Über den Noradrenalin- und ATP-Gehalt sympathischer Nerven. *Arch. exp. Path. Pharmak.* **233**, 296–300.

SCHÜMANN, H. J., SCHMIDT, K. & PHILIPPU, A. (1966). Storage of norepinephrine in sympathetic ganglia. *Life Sci. Oxford* **5**, 1809–1815.

SCHÜMANN, H. J., SCHNELL, K. & PHILIPPU, A. (1964). Subcelluläre Verteilung von Noradrenalin und Adrenalin im Meerschweinchenherzen. *Arch. exp. Path. Pharmak.* **249**, 251–266.

SMITH, A. D. (1968). Biochemistry of adrenal chromaffin granules. *The Interaction of Drugs and Subcellular Components in Animal Cells*, ed. CAMPBELL, P. N., pp. 239–292. London: Churchill.

SMITH, A. D. & WINKLER, H. (1966). The localization of lysosomal enzymes in chromaffin tissue. *J. Physiol.* **183**, 179–188.

SMITH, A. D. & WINKLER, H. (1968). Lysosomal phospholipases A_1 and A_2 of bovine adrenal medulla. *Biochem. J.* **108**, 867–874.

STJÄRNE, L. (1966). Studies of noradrenaline biosynthesis in nerve tissue. *Acta physiol. scand.* **67**, 441–454.

STJÄRNE, L., ROTH, R. H. & GIARMAN, N. J. (1968). Microsomal monoamine oxidase in sympathetically innervated tissue. *Biochem. Pharmac.* **17**, 2008–2012.

TAYLOR, P. W., CHIDSEY, C. A., RICHARDSON, K. C., COOPER, T. & MICHAELSON, I. A. (1966). Subcellular distribution of norepinephrine in the normal and surgically denervated cat heart. *Biochem. Pharmac.* **15**, 681–690.

VIVEROS, O. H., ARQUEROS, L. & KIRSHNER, N. (1968). Release of catecholamines and dopamine-β-oxidase from the adrenal medulla. *Life Sci. Oxford* **7**, 609–618.

WEGMANN, A. & KAKO, K. (1961). Particle bound and free catecholamines in dog hearts and the uptake of injected norepinephrine. *Nature, Lond.* **192**, 978.

WINKLER, H. (1969). Isolierung und Charakterisierung von chromaffinen Noradrenalin-Granula aus Schweine-Nebennierenmark. *Arch. exp. Path. Pharmak.* **263**, 340–357.

WURTMAN, R. J. & AXELROD, J. (1963). A sensitive and specific assay for the estimation of monoamine oxidase. *Biochem. Pharmac.* **12**, 1439–1441.