Biochemical Pharmacology, Vol. 22, pp. 3005–3011. Pergamon Press, 1973. Printed in Great Britain.

ARTIFACTUAL SUBCELLULAR DISTRIBUTION OF DOPAMINE β-HYDROXYLASE

EFFECTS OF DILUTION AND NON-SPECIFIC PROTEIN ON THE DOPAMINE β-HYDROXYLASE ACTIVITY OF MEMBRANES OF ADRENOMEDULLARY AND SYMPATHETIC NERVE VESICLES

HUGO LAGERCRANTZ, LENNART STJÄRNE, TORGEIR FLATMARK and KAREN B. HELLE

Department of Physiology, Karolinska Institute, Stockholm, Sweden
and Departments of Biochemistry and Physiology, University of Bergen, Norway

(*Received* 16 *February* 1972; *accepted* 30 *May* 1973)

Abstract—Dopamine β-hydroxylase (EC 1.14.2.1.) activity showed a bimodal distribution pattern on isopycnic sucrose gradient centrifugation analysis of a crude preparation of membranes of bovine adrenomedullary vesicles. The two peaks of DBH activity corresponded to the peak of catecholamines (CA) and to the peak of the mitochrondrial "marker enzyme" cytochrome *c* oxidase. The high DBH activity of the latter peak was found to be due to stimulation by mitochondrial contamination.

The specific activity of dopamine β-hydroxylase in membranes of highly purified bovine adrenomedullary vesicles was shown to be reduced by approx. 85 per cent upon dilution. This effect of dilution was abolished, and the activity enhanced, by the presence of a mitochondrial fraction. This enhancing effect is probably due partly to nonspecific stimulation of the DBH activity by mitochondrial protein and partly to catalase present in the mitochondrial preparation. A similar effect has been observed with sympathetic nerve vesicles. The implications of this effect for other density gradient studies are discussed.

THE ENZYME dopamine β-hydroxylase (DBH, EC 1.14.2.1) appears to be specific for the catecholamine (CA) storage particles of adrenal medulla[1] and sympathetic nerves.[2] This copper enzyme is found as a constituent of the matrix as well as of the membrane phases of these organelles although the highest specific activities are obtained in fractions derived from the membranes. Thus, DBH activity has been used as a "marker enzyme" of CA storage particles as well as of membrane fragments derived from these organelles in fractionation studies. However, on fractionation of the various subcellular particles of adrenal medulla by density gradient centrifugation, a small, but consistent difference has been reported between the distribution patterns of DBH and CA.[3,4] No satisfactory explanation has so far been given for this discrepancy in the density profile of the two "markers" of adrenergic storage organelles.

The purpose of the present study is to demonstrate that this discrepancy is an artifact, i.e. due to an unspecific stimulation of the DBH activity by contamination with other subcellular particles, notably mitochondria.

MATERIALS AND METHODS

Preparation of subcellular particles

Bovine adrenal chromaffin granules. These were obtained by two methods:

Method 1. A crude preparation of chromaffin granules was prepared by differential

centrifugation of an Ultra-Turrax homogenate of bovine adrenal medulla. Coarse particles were removed by centrifugation in a Servall SS-34 rotor at 6000 g_{max} min and a "large granule" fraction was obtained by a subsequent centrifugation at 3×10^5 g_{max} min. This pellet was resuspended in 30 ml of 20 mM Tris–HCl, pH 7·5 in order to induce hypo-osmotic lysis of the CA vesicles. The membranes of the lysed vesicles as well as contaminating subcellular particles were sedimented at 3×10^5 g_{max} min and the lysis step was repeated once. The pellet thus obtained was resuspended in 0·3 M Tris-buffered sucrose (pH 7·5) to give approx 5 mg of protein/ml; the CA content was 60–90 nmoles/mg of protein. This preparation was used for isopycnic sucrose gradient centrifugation.

Method 2. Highly purified chromaffin CA vesicles were obtained from bovine adrenals as described previously.[5] Lysis was carried out as described above. The procedure was repeated once and the final pellet of washed membrane fragments was resuspended to give approx. 30 mg of protein/ml and was then stored at −20° until used.

Sympathetic nerve vesicles. These were obtained in highly purified form from bovine splenic nerves as previously reported.[6] Vesicles were not lysed prior to use, since about 80 per cent of the DBH activity has been found to be associated with the membrane phase.[7]

Mitochondrial and microsomal *fractions of rat liver homogenates* were prepared essentially as described in the literature.[8]

Analytical methods

Catecholamines. These were analyzed according to the trihydroxyindole method,[9] *protein* with the Folin-Ciocalteu reagent.[10] *Cytochrome* c *oxidase* (EC 1.9.3.1) activity was assayed polarographically[11] and ATP by the fire-fly method.[12]

Dopamine β-hydroxylase (EC 1.14.2.1) activity was determined radiochemically.[13] The reaction mixture contained 100 mM phosphate buffer (pH 6·5), 10 mM ATP, 1 mM ascorbate, 0·5 mM tranylcypromine (Sigma), 0·5 μCi ^{3}H-tyramine (New England Nucl. Corp., Frankfurt a. M.) diluted with non-radioactive tyramine to a final concentration of 5–50 μM, 0–1·0 mM *p*-hydroxymercuribenzoate(PMB) (Sigma), 0·1% (v/v) Triton X-100 (Sigma) and aliquots of enzyme preparations, in a total volume of 1·0 ml. The enzyme reaction was stopped after 15 min of incubation at 37° by addition of 7% (v/v) perchloric acid, and the labelled *p*-hydroxybenzaldehyde formed after oxidation of octopamine was extracted with toluene and measured in a liquid scintillation spectrometer. The enzyme activity of each preparation was measured at three to six different concentrations of the vesicle suspensions and at least two blanks were made for each series by addition of acid before incubation.

RESULTS

Figure 1a (lower curve) demonstrates how the DBH activity of a membrane preparation of adrenomedullary vesicles varies as a function of the particle (protein) concentration. Thus, a linear concentration–activity curve was obtained at a protein concentration of 0·07 mg/ml whereas the specific activity increased as the amount of enzyme is increased above this concentration. On the other hand when the activities were assayed at a constant and optimal concentration of a mitochondrial

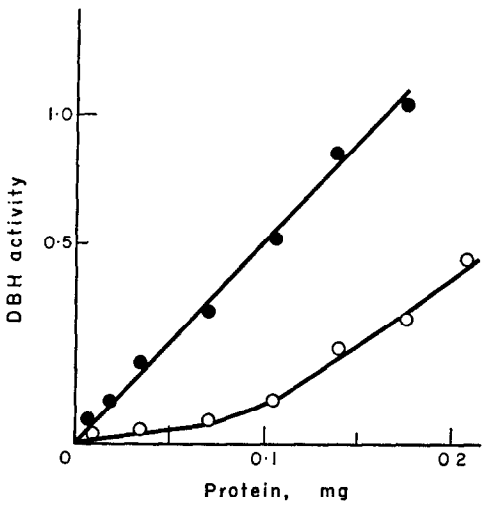

FIG. (1a)

preparation of rat liver, a linear concentration–activity curve was obtained (Fig. 1a, upper curve), and the catalytic activity was greatly enhanced (approximately 10-fold in the low concentration range of the enzyme). This finding suggests that the state of aggregation of the membrane preparation is important for the catalytic activity of DBH and that the inhibitory effect of dilution of the enzyme preparation can be

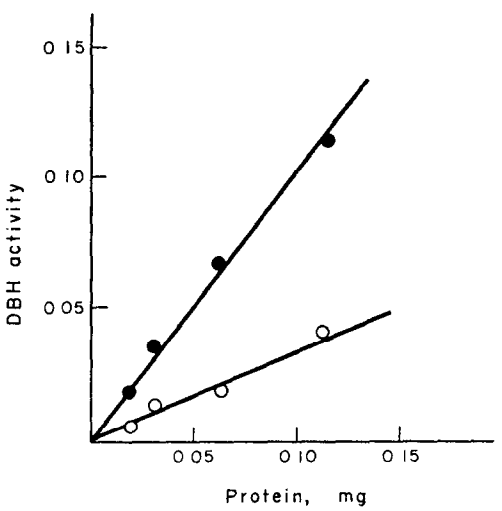

FIG. (1b)

FIG. 1.(a) The relationship between the concentration of the enzyme preparation (adrenomedullary vesicle membranes) and the DBH activity in the absence (○) and presence (●) of a constant and optimal concentration of a liver mitochondrial preparation (0·2 mg of protein/ml). For experimental details, see Methods section; 0·05 mM ^{3}H-tyramine and 1·0 mM PMB were used in the DBH assay. The DBH activity is expressed in nmoles of octopamine formed/min. (b) DBH activity of sympathetic nerve vesicles assayed in the absence (○) and presence (●) of a constant and optimal concentration of a liver mitochondrial preparation (0·2 mg of protein/ml). Experimental conditions as in Fig. 1(a) except that 0·01 mM ^{3}H-tyramine was used in the assay of DBH.

avoided by the presence of a mitochondrial fraction. The DBH activity of the nerve vesicle preparation was found to be less sensitive to dilution and was stimulated to about the same extent as the adrenomedullary enzyme preparation in the higher concentration range (Fig. 1b).

In order to study the nature of the enhancing effect of the mitochondrial preparation, catalase, serum albumin and various subcellular organelles were tested for their effect on DBH activity (Fig. 2). The enhancing effect of the mitochondrial preparation was considerably higher than that of serum albumin but lower than that of catalase. Preliminary experiments showed that lysosomal and microsomal preparations stimulated the DBH activity to about the same extent as serum albumin. A Triton X-100 solubilized preparation of mitochondria was about equally potent as intact mitochondria. Mitochondria even after boiling enhanced the DBH activity (206–242 per cent). Lipid extracts of the mitochondrial preparation and phosphatidylethanolamine also slightly stimulated DBH activity (40–100 per cent), while serum albumin, essentially free of fatty acid, had a higher enhancing effect than plain albumin.

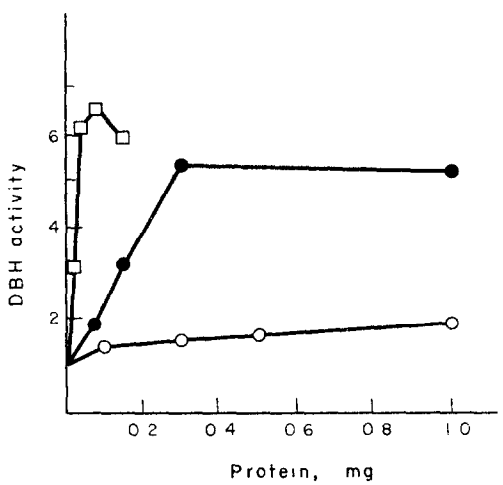

FIG. 2. The effect of varying concentrations of a liver mitochondrial preparation (●), catalase (□) and bovine serum albumin (○) on the DBH activity of a highly purified membrane fraction of adrenal chromaffin granules. Experimental conditions as in Fig. 1 except that PMB was omitted from the incubation mixture. The amount of membrane protein was kept constant at about 0·1 mg/sample.

The enhancing effect of mitochondria might well be due to neutralization of DBH inhibitors. This hypothesis was tested by adding different concentrations of PMB to neutralize inhibitors. Even if PMB had some inhibitory action on the activity of purified DBH it was not found to influence the above described stimulatory effects. In fact the mitochondrial stimulatory effect was slightly enhanced in the presence of PMB.

In some experiments the mitochondrial preparation increased the DBH activity even in the presence of optimal concentrations of catalase. Thus mitochondrial contamination might well cause artifactual distribution patterns of DBH on density gradient analysis of subcellular particles. Figure 3 shows the result of a representative experiment when a crude fraction of chromaffin CA vesicles, subjected to hypo-osmotic

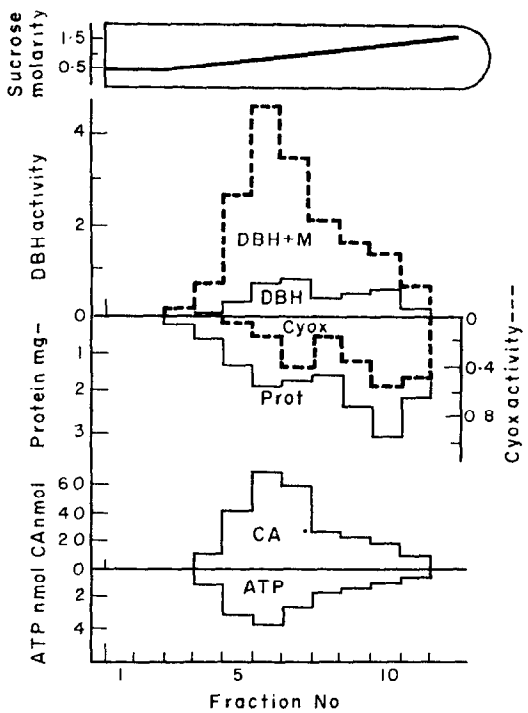

FIG. 3. Distribution of DBH activity, cytochomre *c* oxidase, protein, CA and ATP on isopycnic sucrose gradient centrifugation of a crude preparation of adrenal chromaffin granules subjected to hypo-osmotic lysis. For experimental details, see Methods section and Fig. 1.

lysis, was analyzed by density gradient centrifugation on a linear sucrose gradient. Two bands were formed, at about 0·9 M and 1·3 M sucrose, respectively. CA and ATP occurred largely in the upper band. However, the DBH activity showed a bimodal distribution, as did the protein, with one peak corresponding to CA/ATP and the other one to the lower band, which mainly contained mitochondria as indicated by the distribution of the cytochrome *c* oxidase activity. The possibility that the second DBH peak was artifactual and due to stimulation of the DBH activity of granule membrane fragments by mitochondria was investigated. The DBH activity of each fraction was retested in the presence of a small amount of a mitochondrial preparation isolated from rat liver. Although this mitochondrial preparation was devoid of DBH activity, it induced a five to 10-fold increase in the DBH activity of the fractions of the upper band. The potentiation was marked, but less dramatic, in the lower band, indicating that the DBH activity may have been already submaximally stimulated by other subcellular particles, e.g. mitochondria which represents the major proportion of the particles in these fractions. Thus, the addition of a constant optimal concentration of mitochondria transformed the originally bimodal distribution of the DBH activity to a unimodal (although slightly assymetrical) peak, essentially following that of CA and ATP.

DISCUSSION

The specific activity of DBH in highly purified adrenomedullary vesicle membranes has been shown to be dramatically reduced upon dilution (Fig. 1a), which suggests

that the state of aggregation of the membrane preparation is important for the catalytic activity of this enzyme. This observation lends support to previous assumptions of inactivation of various catalytic activities of these membranes upon dilution;[14,15] it has recently been shown that the NADH: (acceptor) oxidoreductase activities of chromaffin granule membranes are extremely sensitive to dilution.* However, the mechanism by which the mitochondrial fraction stimulates the DBH activity has yet to be elucidated. That the stimulatory effect is partly due to an unspecific effect of solubilized mitochondrial protein is suggested by the fact that albumin alone also stimulates the DBH activity. However, it is already well known that mitochondrial fractions of rat liver homogenate contain some catalase activity,[16-18] and Levin and Kaufman[19] have shown that catalase stimulates the DBH activity of purified preparations of the enzyme.

The implications of this stimulatory effect of mitochondria on the DBH activity of membrane fragments of chromaffin granules and sympathetic nerve vesicles is well illustrated in isopycnic sucrose gradient centrifugation (Fig. 3). Thus, using CA as a marker the peak of chromaffin granule membranes was located at a density of 0·9 M sucrose, and that of mitochondria using cytochrome *c* oxidase as a marker at a density of 1·3 M sucrose. On the other hand, the DBH activity revealed a bimodal distribution pattern, with one peak corresponding to the CA peak and one to the mitochondrial peak. The second peak might suggest that CA vesicle membranes contaminated the mitochondrial fraction. However, as shown by the density profile of the DBH activity in the presence of a small amount of a mitochondrial fraction of rat liver, the bimodal distribution was found to be an artifact.

Our findings may thus explain the discrepancy often observed between the density profiles of CA and DBH activity.[3,4,7] Interactions of the kind observed by us may complicate interpretation of density gradient distributions of DBH, especially in relatively crude tissue homogenate. Even if optimal concentrations of catalase are used there is still a risk that contaminating particles, e.g. mitochondria, may cause artifactual patterns of DBH distribution.

Acknowledgements—We wish to thank Mrs. M. Arvidsson and Mrs. I. Dahlin for skilful technical assistance. This investigation was supported by the Swedish Medical Research Council (B72-99F-3721-01, B72-14X-2479-05).

REFERENCES

1. N. KIRSHNER, *J. biol. Chem.* **226**, 821 (1957).
2. L. STJÄRNE, *Acta physiol. scand.* **76**, 44 (1966).
3. P. LADURON and F. BELPAIRE, *Biochem. Pharmac.* **17**, 1127 (1968).
4. O. H. VIVEROS, L. ARQUEROS, R. J. CONETT and N. KIRSHNER, *Molec. Pharmac.* **5**, 60 (1969).
5. K. B. HELLE, T. FLATMARK, G. SERCK-HANSSEN and S. LÖNNING, *Biochim. biophys. Acta* **226**, 1 (1971).
6. H. LAGERCRANTZ, R. L. KLEIN and L. STJÄRNE, *Life Sci.* **9**, 639 (1970).
7. W. P. DE POTTER, A. D. SMITH and A. F. DE SCHAEPDRYVER, *Tissue and Cell* **2**, 529 (1970).
8. W. C. SCHNEIDER and G. H. HOGEBOM, *J. biol. Chem.* **183**, 123 (1950).
9. U. S. v. EULER and F. LISHAJKO, *Acta physiol. scand.* **51**, 348 (1961).
10. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
11. G. L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER and A. BERGSTRAND, *J. Cell. Biol.* **32**, 415 (1967).
12. B. L. STREHLER and J. R. TOTTER, in *Methods of Biochemical Analysis* (Ed. D. GLICK) Vol. I, p. 341. (1954).

* O. TERLAND and T. FLATMARK, manuscript submitted for publication.

13. O. H. VIVEROS, L. ARQOEROS and N. KIRSHNER, *Life Sci.* **7**, 609 (1968).
14. K. B. HELLE, *Biochim. biophys. Acta* **245**, 94 (1971).
15. T. FLATMARK, O. TERLAND and K. B. HELLE, *Biochim. biophys. Acta* **226**, 9 (1971).
16. H. V. EULER and L. HELLER, *Z. Krebsforsch.* **56**, 393 (1949).
17. A. NYBERG, J. SCHUBERTH and L. ÄNGGÅRD, *Acta chem. scand.* **7**, 1180 (1953).
18. A. FOURCADE and A. J. ROSENBERG, *Bull. Soc. chim. Biol.* **44**, 471 (1962).
19. E. Y. LEVIN and S. KAUFMAN, *J. biol. Chem.* **236**, 2043 (1961).