Mechanism of Secretion from the Adrenal Medulla

III. Studies of Dopamine β-Hydroxylase as a Marker for Catecholamine Storage Vesicle Membranes in Rabbit Adrenal Glands

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SUMMARY

The subcellular distribution of dopamine β-hydroxylase in homogenates of rabbit adrenal glands has been measured to determine the possibility of using the enzyme as a marker to trace the fate of the catecholamine storage vesicle membranes following secretion of adrenaline. Optimal conditions for the assay of the enzyme in each of the subcellular fractions have been determined. These studies show that a large fraction of the total dopamine β-hydroxylase activity of the adrenal glands is present in the 26,000 × *g* supernatant fraction. Most of the enzyme activity associated with the particulate fraction was retained in the particles when they were washed with sucrose. When the particles were washed with distilled water, however, a large proportion of the enzyme activity was obtained in the supernatant fraction but a significant amount remained in the particulate fraction, indicating that the enzyme was contained within vesicles in both a soluble form and a membrane-bound form. The distribution of adrenaline and dopamine β-hydroxylase in a sucrose density gradient showed a peak of dopamine β-hydroxylase activity near the bottom of the gradient closely associated with the adrenaline storage vesicle fraction, and a second peak near the top of the gradient associated with a less dense particulate fraction.

INTRODUCTION

Early studies on the intracellular localization of dopamine β-hydroxylase in the adrenal medulla indicated that the enzyme was almost exclusively associated with the catecholamine storage vesicles (1–3), that it was contained within the vesicles (4), and that it was firmly attached to the membranes of the vesicles (5). These reports suggested that dopamine β-hydroxylase could be used as a marker to trace the fate of the storage vesicle membranes following neurogenically evoked secretion of adrenaline and noradrenaline. Preliminary experiments on the distribution of dopamine β-hydroxylase in the adrenal gland of the cat led to the observations that the adrenal medulla contained potent endogenous inhibitors of the enzyme, and that these inhibitors could be inactivated by heavy metal ions such as Cu^{2+}, Hg^{2+}, and Ag^{+}, or by sulfhydryl-reactive reagents such as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide (6). These studies also showed that a large fraction of the dopamine β-hydroxylase activity of the adrenal gland was readily obtained in the 26,000 × *g* super-

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natant fraction of adrenal homogenates, and that more than half of the dopamine β-hydroxylase activity associated with the storage vesicles was readily released upon lysis with distilled water. The presence of endogenous inhibitors of this enzyme in other tissues has also been reported (7–10).

This report describes the assay and subcellular distribution of dopamine β-hydroxylase in the adrenal gland of the rabbit. These studies were undertaken to determine the feasibility of using this enzyme as a marker for the storage vesicle membranes in rabbits.

METHODS

Preparation of homogenates. Four- to six-month-old male New Zealand white rabbits were used throughout these experiments. The animals were killed by a blow on the back of the head. The adrenal glands were removed, cleansed of external fat and connective tissue, blotted dry, and weighed. The glands were then homogenized in a conical, all glass Potter-Elvehjem homogenizer in 20 volumes of ice-cold 0.3 M sucrose. With the exceptions noted in the accompanying tables and figures, the homogenates were centrifuged at 26,000 × *g* for 20 min, and the supernatant fraction was decanted and saved for assay. The pellet was resuspended in a volume of ice-cold water to give a mixture containing the equivalent of 50 mg of fresh tissue per milliliter. These fractions are designated sucrose supernatant and water particles, respectively.

Assay of dopamine β-hydroxylase. Dopamine β-hydroxylase was assayed by a modification of the method of Friedman and Kaufman (11). Unless otherwise indicated, the reaction mixture contained potassium phosphate buffer, pH 6.0, 100 mM; fumarate, pH 6.0, 10 mM; ATP, pH 6.0, 5 mM; ascorbate, pH 6.0, 1 mM; tranylcypromine, 0.5 mM; ^{3}H-tyramine (generally labeled, 20 μC/μmole), 0.05 mM; and 0.2 ml (equivalent to 10 mg of fresh tissue) of the enzyme preparation. The total volume was 1.0 ml. Because of the stimulatory effect of Cu^{2+}, we routinely assayed the particulate fractions in the presence of 7.5, 10, and 25 μM Cu^{2+}, and the supernatant fractions in the presence of 10, 25, and 50 μM Cu^{2+}. In some experiments *p*-hydroxymercuribenzoate was used at concentrations of 0.75 and 1.0 mM to inactivate the inhibitor(s) present in the supernatant fractions, and at concentrations of 0.5 and 0.75 mM to inactivate the inhibitor(s) present in the particulate fraction. At these concentrations the mercurial was as effective as Cu^{2+} in inactivating the endogenous inhibitors. The maximal values of dopamine β-hydroxylase activity obtained are reported here. The reaction mixtures were incubated for 15 min in 100% oxygen at 37°. The reaction was stopped by the addition of 1.0 ml of 7% perchloric acid, and the octopamine formed was measured as described by Friedman and Kaufman (11).

Monoamine oxidase activity. The monoamine oxidase activity of the fractions was measured by extracting an aliquot of the perchloric acid supernatant under conditions identical with those used for the assay of octopamine, except that periodate was omitted from the reaction mixture.

Sucrose density gradient centrifugations. Linear sucrose density gradients from 1.0 to 2.25 M were prepared in a standard mixing apparatus. Adrenal glands were homogenized in 20 volumes of 0.3 M sucrose and centrifuged at 800 × *g* for 8 min. The sediment was resuspended and saved for assay. The supernatant fraction was centrifuged at 26,000 × *g* for 20 min. The supernatant was decanted and saved for assay, and the sediment, containing the catecholamine storage vesicles, was resuspended in 0.8 ml of 0.3 M sucrose. An aliquot (0.5 ml) of the resuspended vesicles was layered over the sucrose density gradient (4.6 ml) and centrifuged at 48,000 rpm for 3 hr at 5° in a Beckman SW 50 rotor. The bottoms of the tubes were punctured, and 20–21 fractions were collected from each tube and assayed for dopamine β-hydroxylase and adrenaline. Generally the recovery of dopamine β-hydroxylase activity from the gradients was low (30–50%) unless catalase was present in the sucrose solution used to prepare the gradients. From 90 to 100% of the dopamine β-hydroxylase activity was recovered

TABLE 1

Effect of Cu^{2+} on dopamine β-hydroxylase of rabbit adrenal glands

The reaction was carried out as described in METHODS, with the addition of Cu^{2+} noted below. The data were obtained from paired glands of four rabbits. The letters identify the particulate and supernatant fractions from each pair of glands.

Fraction	Experiment	Dopamine β-hydroxylase activity at various Cu^{2+} concentrations					
		0 μM	5 μM	10 μM	25 μM	50 μM	75 μM
		mμmoles octopamine formed/100 mg tissue/hr					
Water particles	a	2.3	14.1	31.9	14.5		
	b	5.7	17.3	49.5	47.0		
	c	3.3	12.4	31.7	30.9		
	d	11.3	18.1	42.9	50.8		
Sucrose supernatant	a	0			26.4	19.5	11.0
	b	0			24.9	23.1	15.5
	c	0		7.3	46.9	52.7	
	d	0		14.5	47.2	45.8	

when the density gradients contained 400 units[3] of catalase per milliliter and the fractions were subsequently assayed in the presence of 400 units of catalase and 0.75 mM *p*-hydroxymercuribenzoate.

Catecholamines. Perchloric acid extracts of the total homogenate and various subcellular fractions were prepared and assayed for catecholamines by the method of von Euler and Lishajko (12) or by the method of Hathaway *et al.* (13). It was not necessary to purify the extracts by absorption on alumina. In the rabbit adrenals, noradrenaline represented 2–5% of the total catecholamines. The total catecholamines are expressed as micrograms of adrenaline.

RESULTS

Effect of Cu^{2+} on dopamine β-hydroxylase activity. A series of studies were carried out to establish the optimal conditions for the assay of dopamine β-hydroxylase in rabbit adrenals. Table 1 shows the effects of different concentrations of Cu^{2+} on enzyme activity. Maximal activity of the water particles was found with Cu^{2+} concentrations of 10 or 25 μM. At higher concentrations of Cu^{2+} the activity was always lower, and with 5 μM Cu^{2+} the activity was generally lower than that at 10 μM Cu^{2+}. Maximal activity in the supernatant fraction was found at Cu^{2+} concentration of 25 or 50 μM; at higher or lower concentrations the activity was usually lower. Only occasionally could we detect activity in the sucrose supernatant in the absence of Cu^{2+}.

Effects of pH, incubation time, and substrate concentration on dopamine β-hydroxylase activity. The effect of pH on

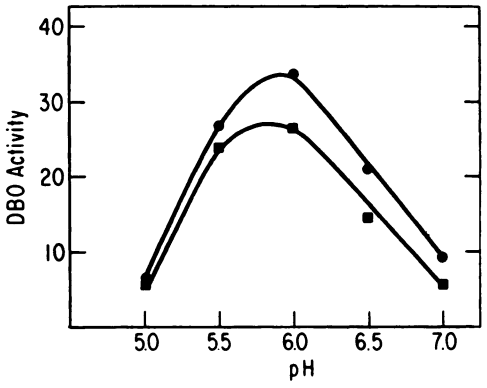

FIG. 1. *Effect of pH on dopamine β-hydroxylase (DBO) activity*

Dopamine β-hydroxylase activity is expressed as nanomoles of octopamine formed per 100 mg of tissue per hour. The assays were performed as described in METHODS, with 50 μM ^{3}H-tyramine as substrate. ●, Dopamine β-hydroxylase activity in 26,000 × *g* pellet; ■, dopamine β-hydroxylase activity in 26,000 × *g* supernatant.

[3] One unit of catalase decomposes 1 μmole of H_2O_2 per minute at pH 7.0 and 25° while the H_2O_2 concentration decreases in the assay system from 10.3 to 9.2 μmoles/ml.

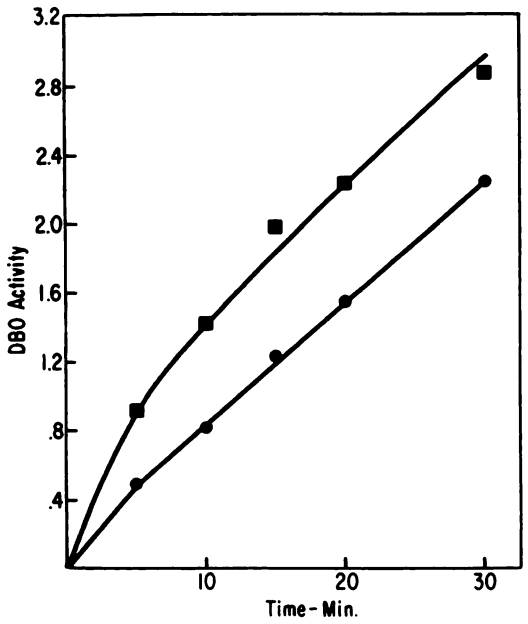

FIG. 2. *Dopamine β-hydroxylase (DBO) activity as a function of incubation time*

Dopamine β-hydroxylase activity is expressed as nanomoles of octopamine formed per 100 mg of tissue during the incubation times shown on the abscissa. The assays were performed as described in METHODS, with 10 μM ^{3}H-tyramine as substrate. ■, Dopamine β-hydroxylase activity in 26,000 × *g* pellet; ●, dopamine β-hydroxylase activity in 26,000 × *g* supernatant.

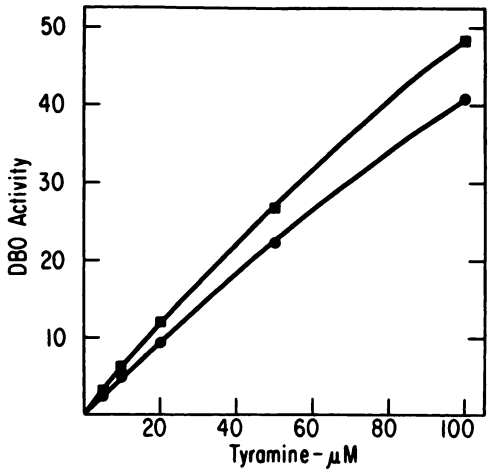

FIG. 3. *Effect of substrate concentration on dopamine β-hydroxylase (DBO) activity*

Dopamine β-hydroxylase activity is expressed as nanomoles of octopamine formed per 100 mg of tissue per hour at the substrate concentrations shown on the abscissa. The assays were performed as described in METHODS. ●, Dopamine β-hydroxylase activity in 26,000 × *g* particulate fraction; ■, dopamine β-hydroxylase activity in 26,000 × *g* supernatant fraction.

dopamine β-hydroxylase activity in the particulate and supernatant fractions is shown in Fig. 1. The optimal pH of 6.0 was found to be the same for both fractions.

The effect of incubation time on the activity of the enzyme is shown in Fig. 2. The reaction was approximately linear for 15 min. This time was chosen for our standard assay conditions.

Figure 3 shows the enzyme activity as a function of substrate concentration. The enzyme activity in the particulate fraction was linear with the concentration of tyramine up to 50 μM; the activity in the supernatant fraction was linear up to a tyramine concentration of 20 μM.

Dopamine β-hydroxylase activity as a function of the amount of tissue present in the incubation mixture is shown in Fig. 4. The enzyme activity was proportional to the amount of tissue present in both the particulate and supernatant fractions in the measured range of 5–15 mg. At the lowest concentration, 2.5 mg, the activity was too low to obtain reliable data. The standard assay conditions in subsequent work contained amounts of the particulate or supernatant fractions equivalent to 10 mg of fresh tissues.

In addition to the above, the effects on the reaction of ascorbate, ATP, fumarate, and catalase were determined. ATP and ascorbate had a slight stimulatory effect when present at 5.0 and 1.0 mM, respectively. When fumarate was present at a concentration of 10 mM, the activity was generally twice that found in the absence of fumarate; the addition of catalase to the supernatant or particulate fractions had no effect on the dopamine β-hydroxylase activity.

Monoamine oxidase activity. Since the particulate fraction of homogenates of the adrenal glands contained monoamine oxidase, it was necessary to inhibit this enzyme because it would oxidize the ^{3}H-tyramine

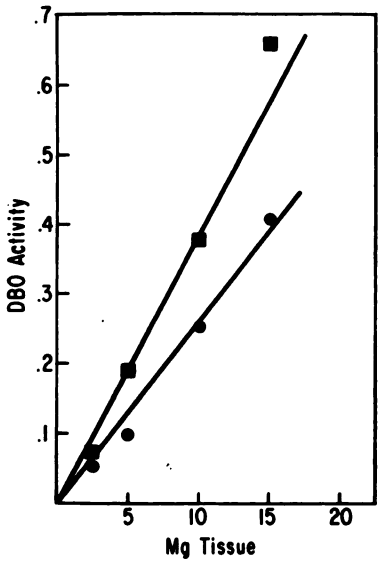

FIG. 4. *Dopamine β-hydroxylase (DBO) activity as a function of tissue concentration*

Dopamine β-hydroxylase activity is expressed as nanomoles of octopamine formed per hour with the different amounts of tissue preparations shown. The assays were performed as described in METHODS, using 10 μM tyramine-^{3}H as substrate. ■, Dopamine β-hydroxylase activity in 26,000 × *g* particulate fraction; ●, dopamine β-hydroxylase activity in 26,000 × *g* supernatant fraction.

to the same product, *p*-hydroxybenzaldehyde, as that formed by the oxidation of octopamine by periodate. Tranylcypromine was used to inhibit the monoamine oxidase activity. As shown in Table 2, tranylcypromine at a concentration of 0.5 mM inhibited the monoamine oxidase activity of the particulate fraction by 95% and had little effect on the dopamine β-hydroxylase activity. Measurement of the latter activity in the absence of tranylcypromine is unreliable, and the data are not included here. With purified dopamine β-hydroxylase from bovine adrenal medulla, however, tranylcypromine in the concentration used here inhibited the enzyme less than 5%. Under our assay conditions, the monoamine oxidase activity was such that the amount of *p*-hydroxybenzaldehyde formed by this activity in the presence of tranylcypromine was less than 5% of the octopamine formed by dopamine β-hydroxylase. The monoamine oxidase activity of the 26,000 × *g* supernatant fraction was only about 10% of that of the particulate fraction and was completely inhibited by 0.25 mM tranylcypromine.

Sucrose gradient centrifugation. The association of dopamine β-hydroxylase with the catecholamine storage vesicles has been demonstrated in the bovine adrenal medulla (1, 3). As shown in Fig. 5, a large fraction of the dopamine β-hydroxylase activity in the particulate fractions of homogenates of rabbit adrenal glands closely paralleled, but did not coincide with, the distribution of adrenaline in sucrose density gradients. Laduron and Belpaire (14) have reported a similar distribution of dopamine β-hydroxylase and catecholamines in bovine adrenal medulla. In this experiment, which is typical of several others, 90% of the dopamine β-hydroxylase activity and 96% of the

TABLE 2

Inhibition of monoamine oxidase in rabbit adrenals by tranylcypromine

Monoamine oxidase and dopamine β-hydroxylase were assayed as described in METHODS, except for the concentration of tranylcypromine. The particulate fractions were assayed in the presence of 10 μM Cu^{2+}, and the supernatant fraction, in the presence of 25 μM Cu^{2+}. The substrate concentration was 10 μM ^{3}H-tyramine.

	Monoamine oxidase activity		Dopamine β-hydroxylase activity	
Tranylcypromine	Particulate	Supernatant	Particulate	Supernatant
mM	*mμmoles product formed/100 mg tissue/hr*			
0	7.63	0.83		
0.10	1.24	0.09	3.58	3.23
0.25	0.66	0.0	3.38	3.40
0.50	0.46	0.0	3.29	3.12

adrenaline applied to the gradient were recovered in the various fractions. Fractions 1–11 contained 67% of the dopamine β-hydroxylase activity and 75% of the adrenaline. Near the top of the gradient, fractions 18–20 contained 17% of the dopamine β-hydroxylase activity and 7% of the total adrenaline. The dopamine β-hydroxylase activity in the latter fractions was presumably due to the enzyme bound to the membranes of disrupted storage vesicles. Since these upper fractions also contained mitochondrial, microsomal, and possibly other types of membrane structures, it is possible that some of the soluble dopamine β-hydroxylase activity present in the initial homogenate may have become attached to these membranes.

The fact that the peak of dopamine β-hydroxylase activity in the lower portion of the gradient did not coincide with the peak of adrenaline indicates two populations of particles. This does not mean that dopamine β-hydroxylase-containing particles are different from adrenaline storage vesicles. The observation shown in Fig. 5 can be explained by the following assumptions.

1. The adrenaline content of the storage vesicles obeys a normal distribution.

2. The density of each vesicle is directly related to its adrenaline content.[4]

3a. Each vesicle contains the same amounts of dopamine β-hydroxylase, or

3b. The dopamine β-hydroxylase content of the vesicles obeys a normal distribution.

From postulates 1 and 2 the distribution of adrenaline in the gradient would not be identical with the distribution of the number of vesicles. It would necessarily follow that fraction 2 (Fig. 5), which contained approximately 6% of the adrenaline, would contain fewer vesicles than the fraction that contained the same amounts of adrenaline on the descending limb of the distribution curve. This, in combination with postulate 3a, would give the observed distribution. Alternatively, if postulates 1, 2, and 3b are correct, the distribution of dopamine β-hydroxylase and adrenaline would coincide only if each vesicle contained the same dopamine β-hydroxylase to adrenaline ratio. The maximum activity observed for dopamine β-hydroxylase, measured under the conditions of these assays, would appear to be potentially sufficient to synthesize the entire adrenaline content of the adrenal gland in approximately 0.15–15 min. Levin *et al.* (5) have calculated that there is sufficient dopamine β-hydroxylase in the bovine adrenal gland to synthesize its entire content of adrenaline and noradrenaline in 0.25 min. Since the adrenaline content of the vesicles is also dependent on the formation of an ill-defined storage complex within the vesicles, it is not unlikely that dopamine β-hydroxylase and adrenaline are contained within the same particles but in different relative amounts and that their distribution in a sucrose gradient is not coincidental. Recent evidence (15) that adrenaline plus noradrenaline and dopamine β-hydroxylase are released from perfused bovine adrenal glands in the same relative amounts as found in the soluble contents of the storage vesicles further supports the concept that the catecholamines and dopamine β-hydroxylase are contained within the same vesicles.

Dopamine β-hydroxylase in storage vesicles. To determine whether the dopamine β-hydroxylase in the particulate fraction and in the 26,000 × *g* supernatant fraction was bound to membranes (Table 3), the following procedure was employed (see Fig. 6).

Adrenal glands from three rabbits were pooled and homogenized in 0.3 M sucrose. The homogenate was divided into two aliquots and centrifuged at 26,000 × *g* for 20 min. The supernatant fractions (A) were pooled, and an aliquot was centrifuged at 100,000 × *g* for 2 hr. The supernatant fraction (C) was decanted and saved, and the pellet (D) was resuspended in a volume of ice-cold water equal to that of the supernatant. One of the 26,000 × *g* pellets was resuspended in 0.3 M sucrose (B), and an aliquot was centrifuged at 26,000 × *g* for

[4] Studies now in progress on the distribution in sucrose density gradients of storage vesicles in various stages of depletion show changes consistent with this postulate.

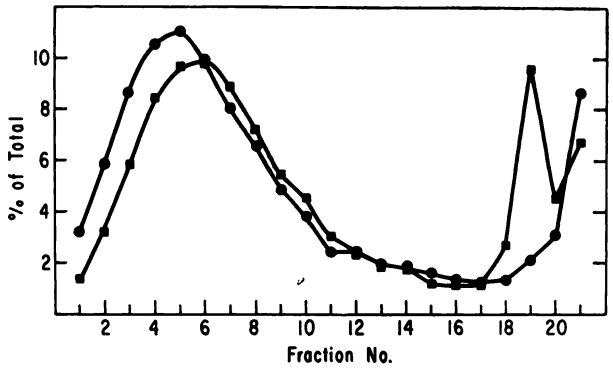

FIG. 5. *Distribution of dopamine β-hydroxylase and adrenaline in a sucrose density gradient*

Dopamine β-hydroxylase activity in the various fractions was assayed in the presence of 400 units of catalase, using 10 μM ^{3}H-tyramine as substrate and 0.75 mM *p*-hydroxymercuribenzoate to inactivate the endogenous inhibitor(s). ●, Adrenaline; ■, dopamine β-hydroxylase.

20 min. The supernatant (E) was decanted, and the pellet was resuspended in water (F). The second pellet obtained from the initial 26,000 × *g* centrifugation was resuspended in distilled water (G), and an aliquot was centrifuged at 26,000 × *g* for 20 min. The sediment was resuspended in distilled water (I), and an aliquot of the supernatant (H) was centrifuged at 100,000 × *g* for 2 hr. The supernatant (J) was decanted, and the pellet was resuspended in distilled water (K).

The data of Table 3 show several interesting aspects of the distribution of dopamine β-hydroxylase in homogenates of the adrenal gland. In this preparation 38% of the maximal total dopamine β-hydroxylase activity (A, H, and I) was obtained in the first 26,000 × *g* sucrose supernatant (A). When this supernatant fraction was centrifuged at 100,000 × *g* for 2 hr, 68% of its total dopamine β-hydroxylase activity was recovered in the soluble fraction (C) and 12% in the pellet (D). Thus 26% of the total dopamine β-hydroxylase activity of the homogenate was obtained in a soluble form in the first supernatant fraction.

The dopamine β-hydroxylase associated with the particulate fraction appears to be largely in solution within the storage ves-

TABLE 3

Subcellular distribution of dopamine β-hydroxylase in homogenates of rabbit adrenal glands

Dopamine β-hydroxylase activity was measured as described in METHODS in the presence of 0.50, 0.75, or 1.0 mM *p*-hydroxymercuribenzoate instead of Cu^{2+}. The substrate concentration was 10 μM ^{3}H-tyramine. The letters correspond to the fractions described in the text and Fig. 6.

Fraction (see Fig. 6)	Dopamine β-hydroxylase	Catecholamines
	mμmoles/100 mg/hr	*μg/100 mg*
A. First 26,000 × *g* sucrose supernatant	3.76	21.6
B. First 26,000 × *g* pellet, resuspended in sucrose	3.12	102.0
C. 100,000 × *g* supernatant of A	2.55	18.6
D. 100,000 × *g* pellet of A	0.44	0.5
E. 26,000 × *g* supernatant of B	0.33	6.6
F. 26,000 × *g* pellet of B	2.30	87.1
G. First 26,000 × *g* pellet, resuspended in water	4.32	93.8
H. 26,000 × *g* supernatant of G	4.76	79.4
I. 26,000 × *g* pellet of G	1.35	12.2
J. 100,000 × *g* supernatant of H	3.50	75.3
K. 100,000 × *g* pellet of H	1.04	0.6

icles. After washing of the 26,000 × *g* pellet (B) in isotonic sucrose, 10% of the enzyme activity was found in the wash (E), and 74% in the pellet (F). When the first 26,000 × *g* pellet (B) was resuspended in water (G) and centrifuged, however, *more* activity was recovered in the supernatant fraction (H) than was initially present in fraction G, and only 22% of the recovered activity (H + I) was found in the pellet (I) while 13% of the total adrenaline was present in this fraction. This indicates that perhaps as much as 50% or more of the dopamine β-hydroxylase activity in fraction I could be removed by additional washings. Of the activity present in fraction H, 74% remained in the 100,000 × *g* supernatant (J) and 22% was recovered in the pellet (K). These data indicate that about 22% of the dopamine β-hydroxylase activity of the storage vesicle is "tightly" bound to the membrane fraction and that approximately 65% of the total dopamine β-hydroxylase activity of the rabbit adrenal gland can be readily solubilized.

DISCUSSION

The data reported herein for the distribution of dopamine β-hydroxylase activity in homogenates of rabbit adrenal glands are quite similar to those reported for bovine adrenal medulla (6). The data of Table 3 indicate that the particulate dopamine β-hydroxylase isolated in isotonic sucrose (E and F compared to H and I) is contained within the storage vesicles and that a considerable portion of the total evident activity is readily solubilized once the vesicles are lysed with distilled water. The source of the dopamine β-hydroxylase in the 26,000 × *g* sucrose supernatant fraction of the homogenate is obscure. Not all the activity could have come from disruption of the storage vesicles during homogenization, since 38% of the total dopamine β-hydroxylase activity but only 16% of the total catecholamines were present in this fraction. Several explanations of this observation may be offered. (a) A fraction of the enzyme may exist in soluble form in the intact cell. (b) Evidence has been presented

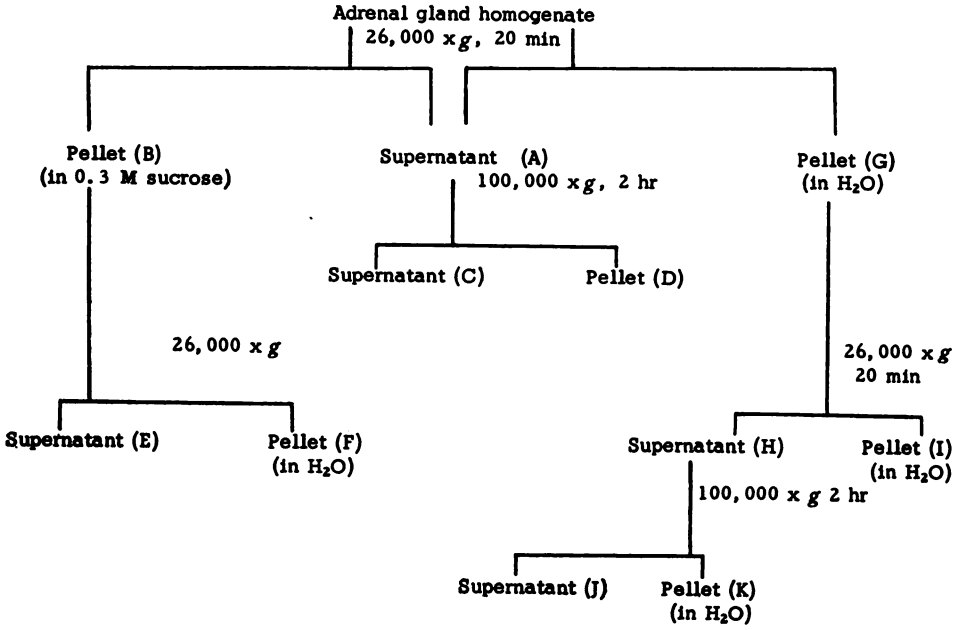

FIG. 6. *Fractionation of adrenal gland homogenates*

The letters in parentheses correspond to the letters in Table 3. See the text for description.

for the existence of two types of storage vesicles (16); it is possible that one vesicle type may have a higher dopamine β-hydroxylase to catecholamine ratio than the other and may be more readily broken. One can speculate that one type may consist of nascent or newly forming vesicles that have their full complement of dopamine β-hydroxylase but have not yet acquired their full complement of catecholamines. (c) A possible though unlikely explanation is that dopamine β-hydroxylase is leached out of the vesicles more readily than the catecholamines; the data of Table 3 (C and D) do not support this. (d) A fourth possibility is that the activity of the enzyme in solution is greater than the activity of the same amount of enzyme when bound to a membrane. The data of Table 3 (G, H, and I) suggest that the activity may be greater in solution but not sufficiently greater to account for all of the activity in fraction A.

Although a large fraction of the dopamine β-hydroxylase in the storage vesicles is readily solubilized, a significant amount remains attached to the particulate fraction. The dopamine β-hydroxylase activity that appears in the particulate fraction in the upper portion of the sucrose gradients may be present in membranes of empty vesicles and might be used as a marker to follow the fate of disrupted storage vesicles following secretion of adrenaline.

The solubility of dopamine β-hydroxylase suggests that the enzyme might be released during secretion in a manner analogous to the release of other soluble proteins contained within the vesicles (17–21). Preliminary experiments with isolated, perfused bovine adrenal glands indeed show that dopamine β-hydroxylase is released during acetylcholine-evoked secretion of catecholamines (15). The observations reported here further suggest that measurements of this enzyme in the particulate and supernatant fractions of adrenal homogenates prepared from control and neurogenically stimulated adrenal glands would provide significant information on the mechanism of secretion and the subsequent fate of the storage vesicles. Such studies have been carried out and will be reported in a following communication.

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