MOLECULAR PHARMACOLOGY, 7, 434–443

Mechanism of Secretion from the Adrenal Medulla

VI. Effect of Reserpine on the Dopamine β-Hydroxylase and Catecholamine Content and on the Buoyant Density of Adrenal Storage Vesicles

O. H. VIVEROS,[1,2] L. ARQUEROS,[1] AND N. KIRSHNER

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27706

(Received February 8, 1971)

SUMMARY

The possibility of differentiating between chromaffin vesicles with different catecholamine contents was tested by studying the distribution of rabbit adrenal dopamine β-hydroxylase (EC 1.14.21) and catecholamines, and the buoyant densities of the catecholamine storage vesicles after isopycnic centrifugation of crude storage vesicle fractions in sucrose density gradients. Catecholamine storage vesicles were prepared from adrenal glands of untreated rabbits, rabbits which had received chlorisondamine chloride (10/kg intraperitoneally) to block ganglionic transmission, and rabbits which had received both chlorisondamine chloride and reserpine (1 mg/kg). Adrenal glands were examined 1 day after treatment with chlorisondamine and 1 and 8 days after combined treatment with chlorisondamine and reserpine. Intact storage vesicles obtained from glands of untreated animals had a specific gravity of 1.27, while the membranes obtained from vesicles lysed in distilled water had a specific gravity of 1.12. Chlorisondamine had no effect on the total dopamine β-hydroxylase and catecholamine content of the adrenal glands or on the buoyant density, even though there was a slight reduction of the activities in segments A and B of the gradients. Twenty-four hours after treatment with chlorisondamine plus reserpine there was a marked decrease in the catecholamine content, no change in the dopamine β-hydroxylase activity, and a significant decrease in the buoyant density of the storage vesicles; the ratio of dopamine β-hydroxylase to catecholamines in the purified storage vesicles was 2–3 times greater than those of untreated and chlorisondamine-treated animals. Eight days after treatment with reserpine and chlorisondamine the dopamine β-hydroxylase activities were twice those of untreated animals, but the catecholamine content was only 60–70% of the control levels. At eight days the buoyant density of the storage vesicles was still significantly less than that of vesicles obtained from untreated rabbits, but not different from that of chlorisondamine-treated animals. These results suggest that recovery of the catecholamine content following reserpine administration requires synthesis of new storage vesicles, but this may not be the srate-limiting step in the recovery. Isopycnic centrifugation through sucrose density gradient provides a method for differentiating normally filled, partially filled, and empty storage vesicles.

This work was supported by Grant AM 05427 from the National Institutes of Health.

[1] Present address, Department of Physiology and Biophysics, University of Chile, Santiago, Chile.

[2] International Fellow of the National Institutes of Health during the tenure of this work.

INTRODUCTION

Secretion from the adrenal medulla occurs by exocytosis, during which the soluble contents of the catecholamine storage vesicles are secreted directly to the exterior of the cell (1–8), leaving the vesicle mem-

branes within the cytoplasm (9). No information has been available to determine whether those vesicles which respond to the secretory stimulus release all or only part of their content. The fact that dopamine β-hydroxylase (EC 1.14.21) is a component of the soluble content of the storage vesicles secreted together with catecholamines and that it is also a component of the membrane fraction retained within the cytoplasm (10–12) suggested an experimental approach to determine whether secretion from each vesicle occurs in an "all-or-none" fashion. If the vesicles secrete their total soluble content, one would expect a decrease in the total dopamine β-hydroxylase and catecholamine contents of the adrenal gland, but the remaining storage vesicles, which do not participate in the secretory response, should have the same properties and catecholamine content as vesicles obtained from unstimulated glands; their dopamine β-hydroxylase to catecholamine ratio and buoyant density should be the same as those of control vesicles. In addition, after stimulation one should find a fraction of particulate dopamine β-hydroxylase corresponding to the membranes of "emptied" vesicles. On the other hand, if vesicles release only a portion of their content, one would expect a decrease in the total dopamine β-hydroxylase content of the gland, an increase in the dopamine β-hydroxylase to catecholamine ratio of the isolated vesicles, and a change in their buoyant density if the density is related to the vesicle content.

Highly purified adrenal catecholamine storage vesicles can be readily obtained by centrifugation through sucrose density gradients (13–15). The studies reported here describe the distribution of dopamine β-hydroxylase and catecholamines in sucrose density gradients, and the buoyant properties of storage vesicles and storage vesicle membranes obtained from adrenal glands of untreated animals and from adrenal glands of animals treated with reserpine under conditions which prevent neurogenically evoked secretion but still cause depletion of the catecholamine content unrelated to secretion. The results show that storage vesicles obtained from reserpine-treated animals have a normal dopamine β-hydroxylase content, a lower catecholamine content, and a lighter buoyant density. A subsequent communication will describe the effects of neurogenic stimulation on the properties of the storage vesicles. A preliminary report of this work has been published (16).

METHODS

Treatment of animals. All animals were a strain of New Zealand white rabbits and weighed 2–3 kg. Chlorisondamine chloride, a long-lasting ganglionic blocking agent, was administered to prevent neurogenic stimulation of the adrenal gland as an indirect effect of reserpine administration (12). Reserpine (1 mg/kg) was administered via the ear vein; chlorisondamine chloride (10 mg/kg) was injected intraperitoneally. Animals which received both reserpine and chlorisondamine were treated with the latter 1 hr before administration of reserpine. Animals which received only chlorisondamine were killed 25 hr after treatment; those which received reserpine or reserpine plus chlorisondamine were killed 15 min, 24 hr, or 8 days after reserpine treatment.

Preparation of homogenates for sucrose density centrifugation. The rabbits were killed by a blow on the base of the skull. The adrenal glands were removed immediately and placed in ice-cold 0.3 M sucrose. The glands were cleaned of fat and connective tissue, blotted dry, weighed and homogenized in 20 volumes of ice-cold 0.3 M sucrose, using conical all-glass Potter-Elvehjem homogenizers. The homogenate was centrifuged at 800 × *g* for 10 min. The pellet was discarded, and the supernatant fluid was centrifuged at 26,000 × *g* for 20 min. The 26,000 *g* pellet (crude storage vesicle fraction) was gently resuspended in 0.8 ml of 0.3 M sucrose. An aliquot was removed for measurement of total dopamine β-hydroxylase and catecholamines in the storage vesicle fraction, and 0.5 ml of the remainder was layered over the sucrose density gradients described below. To prepare lysed vesicles, the 26,000 × *g* sediment was rehomogenized in 10 ml of ice-cold distilled water and centrifuged at 26,000 × *g* for 20 min. The pellet was resuspended in 0.8 ml of 0.3 M sucrose, and 0.5 ml was layered over the sucrose gradients.

Sucrose density gradients. Approximately linear sucrose density gradients of two different concentration ranges were used in these studies: heavy sucrose density gradients ranging in concentration from 2.25 to 1.0 M, and light density gradients ranging in concentration from 1.5 to 0.3 M. The gradients were prepared in a standard mixing apparatus (11). The mixing chamber contained 2.3 ml of the denser sucrose solution, and the reservoir contained 2.3 ml of the lighter sucrose solution. A cushion of 0.3 ml of 2.25 M sucrose was added to the centrifuge tubes for the Spinco SW 50 rotor before forming the gradient. Each of the sucrose solutions contained 300 units of catalase (Sigma, beef liver, twice crystallized) per milliliter. The crude storage vesicle preparation (0.5 ml in 0.3 M sucrose) was layered over the gradient and centrifuged at 48,000 rpm for 3 hr at 5°. This was sufficient time for the vesicles to equilibrate at their buoyant densities (11). The bottoms of the tubes were punctured, and 19–21 fractions containing 12 drops each were collected and assayed for dopamine β-hydroxylase and catecholamines. The specific gravities of the fractions were determined by measuring the refractive index in an Abbé refractometer. Catecholamines and dopamine β-hydroxylase were assayed as previously described (9). Monoamine oxidase was assayed as described by Laduron and Belpaire (15), using ^{3}H-tyramine as substrate.

Materials. Reserpine (Serpasil) and chlorisondamine chloride (Su 3088) were obtained from Ciba Pharmaceutical Company. Chlorisondamine was prepared for injection by suspending 10 mg/ml in 0.9% NaCl. ^{3}H-Tyramine was obtained from New England Nuclear Corporation and was purified before use by adsorption on a column (0.5 × 3 cm) of Dowex 50-H$^+$, followed by elution with 0.5 N HCl.

RESULTS

Effects of chlorisondamine and chlorisondamine plus reserpine on storage vesicles. The crude storage vesicle fractions obtained from adrenal glands of control and drug-treated rabbits were resuspended in 0.3 M sucrose and centrifuged through heavy sucrose density gradients. Fractions were collected and assayed for catecholamines and dopamine β-hydroxylase as described in METHODS. Figure 1 and Table 1 present the data obtained. Because of small differences in the size of the drops from different gradients, the total number of fractions collected varied from 19 to 21. The volume of each fraction was approximately 0.25 ml. For analysis the data were grouped into five segments of the gradient. Segment E consisted of the two uppermost fractions and was equivalent to the volume of material placed on the gradient. Segment D contained the next three fractions. The four fractions below this were grouped in segment C, and consisted of the low plateau of activity intermediate between the two major peaks of dopamine β-hydroxylase activity. The remaining 10–12 fractions, which contained the purified storage vesicles, were divided equally into segments A and B. To define the positions of the denser peaks of dopamine β-hydroxylase and catecholamines in the gradients, the number of fractions from the peaks of dopamine β-hydroxylase and of catecholamines to the top of the gradient was divided by the total number of fractions in the gradient. This is designated R_g, and is a relative measure of the buoyant equilibrium positions.

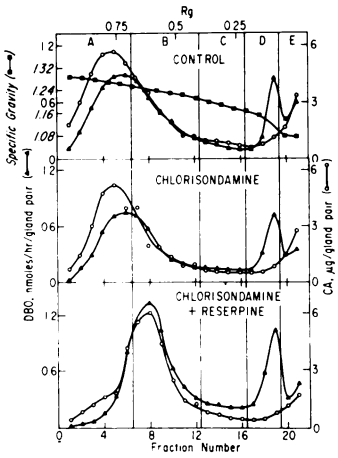

FIG. 1. *Distribution of dopamine β-hydroxylase (DBO) and catecholamines (CA) after centrifugation of crude storage vesicle fraction through "heavy" sucrose density gradients*

Treatment with chlorisondamine alone had no effect on total dopamine β-hydroxylase and catecholamines or on the positions of the peaks. Twenty-four hours after treatment with chlorisondamine plus reserpine there was no change in total dopamine β-hydroxylase (9), but there was a decrease in the catecholamine content and a significant shift of the dopamine β-hydroxylase and catecholamine peaks to a less dense position in the gradient compared with the peaks obtained either from untreated animals or from animals treated only with chlorisondamine. This was also shown by a decrease in the dopamine β-hydroxylase in segment A, no change in segment B, and increases in segments D and E. These changes reflect the appearance of vesicles of lighter density, and of empty vesicle membranes, in segment D. The catecholamine levels in all segments of the gradients were significantly below the control values, resulting in a marked elevation of the dopamine β-hydroxylase to catecholamine ratios.

The data in Fig. 1 are from a single experiment. The adrenals of reserpine-treated animals were among those apparently least depleted, and had a total catecholamine content and total dopamine β-hydroxylase activity of 35 μg/gland pair and 11.1 nmoles/hr/gland pair, respectively, compared to the lowest values shown by control animals, which had a catecholamine content of 52 μg/gland pair and dopamine β-hydroxylase activity of 9.1 nmoles/hr/gland pair.

Eight days after treatment with chlorisondamine plus reserpine the dopamine β-hydroxylase activity was approximately twice that of either the untreated animals or those treated with chlorisondamine, but the catecholamine content had still not recovered to the levels of the animals treated only with chlorisondamine. The buoyant equilibrium positions of the catecholamine and dopamine β-hydroxylase peaks at 8 days were still displaced upward in the gradient compared to those of the untreated animals, but were not significantly displaced from those of the chlorisondamine-treated animals. The dopamine β-hydroxylase activity in segment A was within control levels, but the enzyme activities in all the other segments were 2–3 times greater than the controls. At the same time the catecholamine levels in all but segment B were still below normal levels. This combination of events also resulted in marked increases in the dopamine β-hydroxylase to catecholamine ratios in all segments of the gradient.

Two animals were examined in a similar manner 15 min after treatment with reserpine to determine whether reserpine itself caused a decrease in buoyant density of the storage vesicles. At this time the uptake of catecholamines by the isolated vesicles is completely blocked, but there are no changes in the dopamine β-hydroxylase and catecholamine contents (12). The distribution patterns of storage vesicles obtained from these animals were not different in any manner from those of the controls.

Distribution of membrane-bound dopamine β-hydroxylase in sucrose gradients. To obtain additional information on the source of dopamine β-hydroxylase in segment D, the storage vesicles obtained by centrifugation of adrenal homogenates at 26,000 × *g* were lysed by suspension in distilled water. The particulate fraction was sedimented by centrifugation, washed once with distilled water, resuspended in 0.3 M sucrose, and centrifuged through a heavy sucrose density gradient. All the dopamine β-hydroxylase was found in a well-defined fraction (Fig. 2) which equilibrated at the same specific gravity as fraction D. When purified dopamine β-hydroxylase, or the soluble dopamine β-hydroxylase present in a 100,000 × *g* supernatant fraction of lysed storage vesicles, was centrifuged through the sucrose density gradients, all the activity was recovered in the two uppermost fractions (segment E). Poisner *et al.* (17) found increased amounts of phospholipid in portions of sucrose density gradients less dense than the storage vesicles after stimulation of isolated bovine adrenal glands with acetylcholine, and attributed this to the presence of storage vesicle membranes.

Monoamine oxidase activity in sucrose density gradients. In some experiments in which the crude vesicle fractions were centrifuged through heavy sucrose density gradients,

TABLE 1

Distribution of dopamine β-hydroxylase and catecholamines in segments of sucrose density gradients

p values were calculated by Student's *t*-test relative to the control group.

Treatment	Dopamine β-hydroxylase activity						R_g
	A	B	C	D	E	Total	
	nmoles × 100/gland pr/hr						
None (7)[a]	412 ± 46	441 ± 46	66 ± 2.4	126 ± 8.4	90 ± 8.5	1130 ± 89	0.74 ± 0.01
Chlorisondamine (7)	289 ± 19[b]	320 ± 26[b]	67 ± 10	125 ± 14	78 ± 9.7	904 ± 62	0.74 ± 0.02
Chlorisondamine + reserpine (24 hr) (7)	134 ± 22[c]	403 ± 37	147 ± 23[d]	249 ± 25[c]	92 ± 13	1025 ± 69	0.64 ± 0.01[c]
Chlorisondamine + reserpine (8 days) (4)	382 ± 58	881 ± 156[e]	215 ± 37[c]	369 ± 81[c]	210 ± 58[b]	2047 ± 344[e]	0.68 ± 0.01[f]
Treatment	Catecholamines						R_g
	A	B	C	D	E	Total	
	μg/gland pr						
None (7)	32.1 ± 4	25.9 ± 3.4	5.5 ± 0.9	4.0 ± 0.4	6.7 ± 0.8	74.3 ± 9.0	0.79 ± 0.01
Chlorisondamine (7)	26.3 ± 3.2	19.7 ± 2.3	3.5 ± 0.4	2.2 ± 0.3[f]	4.9 ± 0.9	59.5 ± 6.9	0.75 ± 0.02
Chlorisondamine + reserpine (24 hr) (7)	6.5 ± 1.5[c]	10.2 ± 2.1	1.7 ± 0.3[g]	1.3 ± 0.2[c]	1.9 ± 0.3[c]	21.6 ± 4.2[c]	0.68 ± 0.01[c]
Chlorisondamine + reserpine (8 days) (4)	14.9 ± 1.9[d]	21.5 ± 3.4	3.6 ± 0.5	2.3 ± 0.3[d]	3.0 ± 0.4[c]	45.3 ± 6.0[b]	0.71 ± 0.02[f]

Treatment	Dopamine β-hydroxylase to catecholamine ratio					
	A	B	C	D	E	Total
	nmoles × *100/hr/μg*					
None (7)	13.5 ± 1.7	17.6 ± 1.4	13.6 ± 1.8	33.9 ± 4.6	13.7 ± 1.2	15.8 ± 1.4
Chlorisondamine (7)	11.9 ± 1.4	17.6 ± 2.7	20.4 ± 3.7	59 ± 7[d]	20 ± 4.7	16.7 ± 2.5
Chlorisondamine + reserpine (24 hr) (7)	24.1 ± 3.8[b]	52.3 ± 12.9[b]	106 ± 26[c]	238 ± 61	55.4 ± 11	62.5 ± 15[e]
Chlorisondamine + reserpine (8 days) (4)	25.9 ± 1.3[c]	42.9 ± 7.0[c]	61.3 ± 8.3[c]	155 ± 17[c]	66.4 ± 8.4[c]	49.0 ± 11.5[f]

[a] Numbers in parentheses denote the number of animals.
[b] $p < 0.05$.
[c] $p < 0.001$.
[d] $p < 0.02$.
[e] $p < 0.01$.
[f] $p < 0.005$.
[g] $p < 0.002$.

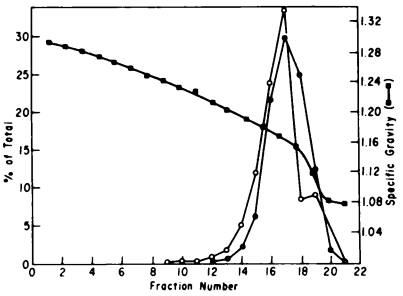

FIG. 2. *Distribution of dopamine β-hydroxylase and monoamine oxidase present in water-insoluble residue of lysed crude storage vesicle fraction after isopycnic centrifugation in "heavy" sucrose density gradients*

●——●, dopamine β-hydroxylase; ○——○, monoamine oxidase.

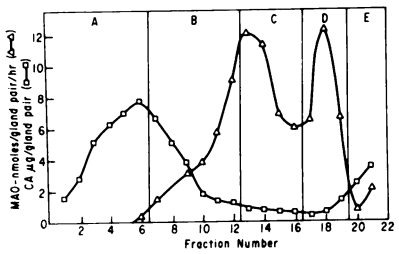

FIG. 3. *Distribution of monoamine oxidase (MAO) and catecholamines (CA) after isopycnic centrifugation of crude storage vesicle fractions through "heavy" sucrose density gradients*

an aliquot of each fraction was assayed for monoamine oxidase to determine the efficacy with which the gradients separated storage vesicles from mitochondria and to rule out the possibility that monoamine oxidase might have interfered with the determination of dopamine β-hydroxylase. Two peaks of activity were found (Fig. 3). One peak equilibrated between the two major peaks of dopamine β-hydroxylase activity, and the peak of lighter density corresponded with fraction D. When the crude vesicle fraction was lysed and washed in distilled water before application to the sucrose gradient, all the activity was recovered in a single peak of lighter density, corresponding to fraction D (Fig. 2). Two peaks of monoamine oxidase activity have been reported in sucrose density gradient centrifugations of other tissues (18–22). The monoamine oxidase in the denser fraction is associated with mitochondria. The monoamine oxidase in the less dense fractions can be separated from the norepinephrine storage particles of sympathetically innervated tissues, and may be attributed to fragmentation of the outer mitochondrial membrane (19, 20). It should be noted that monoamine oxidase was assayed as described in METHODS, but when the enzyme was incubated under the conditions for the assay of dopamine β-hydroxylase no monoamine oxidase activity was detectable in any of the fractions.

Sedimentation of intact and lysed storage vesicles in light sucrose density gradients. In attempts to resolve further the peaks of catecholamines, dopamine β-hydroxylase, and monoamine oxidase, light sucrose density gradients prepared as described in METHODS were employed. Figure 4 shows the pattern obtained when the intact, crude vesicle fraction was sedimented through these gradients. Vesicles obtained from control animals showed a single peak of catecholamines and dopamine β-hydroxylase, with a pronounced shoulder of dopamine β-hydroxylase activity. In some instances the shoulder was more clearly resolved. The buoyant density of the peak of the major fraction was very similar to that found in heavy sucrose density gradients. The distribution of catecholamines closely

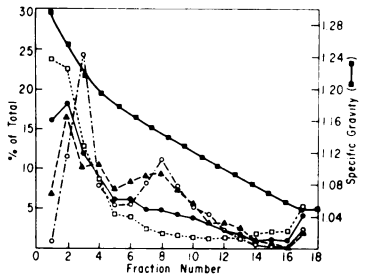

FIG. 4. *Isopycnic centrifugation of crude storage vesicle fraction through "light" sucrose density gradients*

□- - -□, catecholamines, controls; ●——●, dopamine β-hydroxylase, controls; ▲- - -▲, dopamine β-hydroxylase, vesicles prepared 3 hr after insulin treatment (23); ○-·-·○, monoamine oxidase, controls. The data from the insulin-treated animals are included here because the lighter peak of dopamine β-hydroxylase is more prominent than that of the controls.

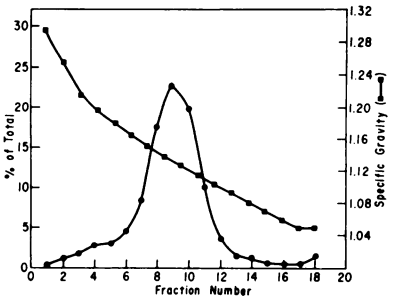

FIG. 5. *Isopycnic centrifugation, on "light" sucrose density gradients, of water-insoluble residue obtained after lysis of crude storage vesicle fraction in distilled water*

The distribution of monoamine oxidase was very similar to that of dopamine β-hydroxylase.

paralleled the distribution of dopamine β-hydroxylase. Two peaks of monoamine oxidase activity were also found, which corresponded to those obtained in the dense gradients. In both heavy and light gradients the position of the peak tube was not exactly reproducible in separate experiments, but the variation was never greater than one fraction. It was not feasible to measure the specific gravity of each fraction in all experiments. The curve showing the specific gravity of both the light and dense gradients is the average obtained from several experiments. In these measurements, the specific gravity of corresponding fractions from different experiments was never displaced more than one fraction.

When the particulate fraction obtained upon lysis of the 26,000 × *g* sediment was centrifuged through the light sucrose density gradients, all the dopamine β-hydroxylase activity was obtained in a single peak (Fig. 5) with a buoyant density corresponding to that of the shoulder of activity observed in Fig. 4, and the same as the buoyant density of the peak of activity in segment D of the heavy sucrose density gradients.

DISCUSSION

It has been shown previously (9) that treatment with reserpine after ganglionic blockade results in depletion of the catecholamine content of the adrenal gland but causes no loss of soluble or particulate dopamine β-hydroxylase. The present studies demonstrate that this drug treatment also leads to a significant decrease in the buoyant density of the storage vesicles when centrifuged through a continuous sucrose density gradient.

The buoyant density of the storage vesicles in concentration gradients is dependent on the intrinsic density of the vesicles themselves, on the density of the solvent, and on the osmolarity of the external medium (22, 24). The internal osmolarity of the storage vesicles is close to 250 milliosmoles (22). In glycogen gradients prepared in water containing different concentrations of sucrose, the density of the vesicles measured at equilibrium varied between 1.12 g/ml in 0.25 M sucrose to 1.23 g/ml in 1.55 M sucrose. The equilibrium density in sucrose gradients prepared in H_2O was 1.23 g/ml, but in sucrose gradients prepared in D_2O the density was 1.27 g/ml (22). Lagercrantz *et al.* (24) obtained similar results in self-generating silicon gradients prepared in aqueous solutions containing different concentrations of sucrose. The increase in density of the storage vesicles in hypertonic solutions is attributed to dehydration and shrinkage as well as to equilibration with the denser sucrose solutions.

The decrease in buoyant density of the storage vesicles after reserpine treatment appears to be related to the loss of catecholamines and ATP. Although ATP was not measured in these experiments, others (25, 26) have demonstrated a loss of both ATP and catecholamines after reserpine treatment. The decrease in density does not appear to be associated with the loss of soluble proteins (chromogranin and soluble dopamine β-hydroxylase activity) from the storage vesicles. Since there were no losses of dopamine β-hydroxylase (mol wt 290,000), it is unlikely that there were losses of chromogranins (mol wt 80,000 for chromogranin A, which, however, has a hydrodynamic radius approaching that of dopamine β-hydroxylase). The decrease in buoyant density also does not appear to be due directly to binding of reserpine itself; vesicles obtained 15 min after treatment with reserpine, when incorporation of exogenous catecholamines by the storage vesicles is maxi-

mally inhibited, had the same density, dopamine β-hydroxylase activity, and catecholamine content as vesicles obtained from untreated animals.

In the experiments described here only the 26,000 × *g* crude vesicle fraction was analyzed on the sucrose density gradients. However, in all groups of animals this fraction contained 70–80% of the total catecholamine and dopamine β-hydroxylase content of the adrenal glands.

Dividing the sucrose density gradients into five segments provides a convenient method for interpreting the results of drug treatment on the properties of the storage vesicles. The purified storage vesicles were present in segments A and B and contained 75–80% of the catecholamines applied to the gradient for all groups of animals. These fractions also contained 75% of the dopamine β-hydroxylase from the control animals and 50–65% of the enzyme from drug-treated animals. Segment C, the portion of the gradient between the two peaks of dopamine β-hydroxylase activity, contained 6–7% of both dopamine β-hydroxylase and catecholamines for the control and chlorisondamine-treated groups; for the reserpine-treated groups this segment contained 8% of the catecholamines and 11–14% of the dopamine β-hydroxylase, the latter change reflecting a larger proportion of partially depleted vesicles or contamination with vesicle membranes. Segment D contained 4–6% of the catecholamines from all groups of animals and 11–14% of the dopamine β-hydroxylase from the control and chlorisondamine-treated groups; in the reserpine-treated groups this segment contained 18–24% of the enzyme activity. The enzyme activity in segment D represents the activity associated with storage vesicle membranes or membrane fragments, since membranes obtained from lysed vesicles also equilibrate in this segment (Fig. 2); the activity from untreated animals can be attributed largely to disruption of the storage vesicles during the preparative procedures. The increased activity in segment D after reserpine treatment, on both an absolute and a relative basis, most likely represents the accumulation of membranes of vesicles which had been completely depleted of their content. The increased activity in segment D from reserpine-treated animals was not due to an effect of reserpine on the fragility of the storage vesicles, since the crude vesicle fraction and fraction E contained the same percentage of total catecholamines and dopamine β-hydroxylase in all groups of animals. Segment E represents the solubilized catecholamines and dopamine β-hydroxylase applied to the gradient. This segment contained 7–9% of the catecholamines and 8–10% of the dopamine β-hydroxylase for all groups of animals, and most probably arose from disruption of the vesicles during the final homogenization procedure.

Eight days after treatment with chlorisondamine and reserpine the dopamine β-hydroxylase activity of the adrenal glands doubled, but the catecholamine content was only 60% of the control levels. The percentage of total catecholamines and dopamine β-hydroxylase in each of the segments of the gradient at 8 days was essentially the same as that at 1 day after reserpine, even though there was a 2–3-fold increase in the total amounts of catecholamines and dopamine β-hydroxylase. This suggests that new vesicles are synthesized while the "old" vesicles are retained. However, synthesis of new vesicles may not be the rate-limiting step in recovery of the catecholamine stores. Doubling of the dopamine β-hydroxylase activity suggests that the number of vesicles initially present in the gland had also doubled. This assumes that each vesicle contains the same amount of enzyme activity, that the observed increase in activity was due to the synthesis of "new" enzyme, and that no "new" enzyme was associated with the old vesicles. If these assumptions are true, the rate-limiting step in recovery of catecholamine stores may be the biosynthesis of catecholamines, or recovery of the ability to store them.

Isopycnic centrifugation through sucrose density gradients and determination of the distribution of catecholamines and dopamine β-hydroxylase provide a method for differentiating normally filled, partially filled, and empty storage vesicles or their membrane fragments. The studies reported here demonstrate that treatment with reserpine after

ganglionic blockade causes, on the whole, a uniform loss of catecholamines from all the storage vesicles but no loss of dopamine β-hydroxylase activity, resulting in an increase in the dopamine β-hydroxylase to catecholamine ratios of the storage vesicles and a decrease in their buoyant densities. Similar studies of the crude storage vesicle fraction following neurogenic stimulation of the adrenal gland are reported in a companion paper (23).

REFERENCES

1. W. W. Douglas, A. M. Poisner and R. P. Rubin, *J. Physiol. (London)* **179**, 130 (1965).
2. W. W. Douglas and A. M. Poisner, *J. Physiol. (London)* **183**, 236 (1966).
3. P. Banks and K. Helle, *Biochem. J.* **97**, 40c (1965).
4. N. Kirshner, H. J. Sage, W. J. Smith and A. G. Kirshner, *Science* **154**, 529 (1966).
5. N. Kirshner, H. J. Sage and W. J. Smith, *Mol. Pharmacol.* **3**, 254 (1967).
6. F. H. Schneider, A. D. Smith and H. Winkler, *Brit. J. Pharmacol. Chemother.* **31**, 94 (1967).
7. H. Blaschko, R. S. Comline, F. H. Schneider, M. Silver and A. D. Smith, *Nature* **215**, 58 (1967).
8. O. H. Viveros, L. Arqueros and N. Kirshner, *Life Sci.* **7**, 609 (1968).
9. O. H. Viveros, L. Arqueros and N. Kirshner, *Mol. Pharmacol.* **5**, 342 (1969).
10. D. S. Duch, O. H. Viveros and N. Kirshner, *Biochem. Pharmacol.* **17**, 255 (1968).
11. O. H. Viveros, L. Arqueros, R. J. Connett and N. Kirshner, *Mol. Pharmacol.* **5**, 60 (1969).
12. O. H. Viveros, L. Arqueros, R. J. Connett and N. Kirshner, *Mol. Pharmacol.* **5**, 69 (1969).
13. N. Kirshner, *Pharmacol. Rev.* **11**, 350 (1959).
14. M. Oka, K. Kajikawa, I. Ohuchi, H. Yoshida and R. Imaizumi, *Life Sci.* **6**, 461 (1967).
15. P. Laduron and F. Belpaire, *Biochem. Pharmacol.* **17**, 1127 (1968).
16. O. H. Viveros, L. Arqueros and N. Kirshner, *Science* **165**, 911 (1969).
17. A. M. Poisner, J. M. Trifaro and W. W. Douglas, *Biochem. Pharmacol.* **16**, 2101 (1967).
18. R. H. Roth and L. Stjärne, *Acta Physiol. Scand.* **68**, 342 (1966).
19. B. Jarrott and L. L. Iversen, *Biochem. Pharmacol.* **17**, 1619 (1968).
20. L. Stjärne, R. H. Roth and N. J. Giarman, *Biochem. Pharmacol.* **17**, 2008 (1968).
21. J. De Champlain, R. A. Mueller and J. Axelrod, *J. Pharmacol. Exp. Ther.* **166**, 339 (1969).
22. P. Laduron, *Studies of dopamine β-hydroxylase.* Doctoral dissertation, Heymans Institute, Rijksuniversiteit, Ghent, 1969.
23. O. H. Viveros, L. Arqueros and N. Kirshner, *Mol. Pharmacol.* **7**, 444 (1971).
24. H. Lagercrantz, H. Pertoft and L. Stjärne, *Acta Physiol. Scand.* **78**, 561 (1970).
25. N.-Å. Hillarp, *Acta Physiol. Scand.* **49**, 376 (1960).
26. N.-Å. Hillarp, *Nature* **187**, 1032 (1960).