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Mechanism of Secretion from the Adrenal Medulla

VII. Effect of Insulin Administration on the Buoyant Density, Dopamine β-Hydroxylase, and Catecholamine Content 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

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SUMMARY

The distribution of dopamine β-hydroxylase (EC 1.14.21) and catecholamines in the adrenal glands of rabbits has been determined by differential and isopycnic centrifugation at various times after treatment with insulin. Fractionation of the subcellular components by differential centrifugation demonstrated that all the dopamine β-hydroxylase bound to the storage vesicle membrane was retained 3 hr after insulin-induced secretion and that only the soluble dopamine β-hydroxylase within the storage vesicle matrix was released. Twenty-four hours after insulin treatment there were changes in the distribution of the membrane-bound enzyme which suggest that partial restoration of storage vesicles had already occurred and that at least some of the membranes of the evacuated vesicles were destroyed.

Isopycnic centrifugation of the crude storage vesicle fraction obtained from adrenal glands 3 hr after insulin administration showed marked decreases in total dopamine β-hydroxylase and catecholamine content but no changes in their relative amounts in those fractions of the sucrose density gradient containing intact storage vesicles and no changes in the buoyant densities of the storage vesicles. These studies indicate that those vesicles which participated in the secretory response released their entire soluble content in an "all-or-none" fashion.

Twenty-four hours after insulin treatment both the dopamine β-hydroxylase and catecholamine content of the glands was still markedly depleted. The buoyant density of the storage vesicles was less than that of vesicles obtained from adrenal glands of untreated rabbits, but the dopamine β-hydroxylase to catecholamine ratio of the vesicles was the same as that of controls. Forty-eight hours after insulin treatment the dopamine β-hydroxylase levels had recovered to normal but the catecholamine content was only 35% of control values. The buoyant density of the storage vesicles suggested that there was a larger proportion of vesicles with lighter than normal densities, and the dopamine β-hydroxylase to catecholamine ratio was 3 times normal values. Glands examined 96–144 hr after insulin treatment were indistinguishable from glands of untreated animals. These studies indicate that during recovery the formation of storage vesicles is not the rate-limiting step, and that there is a considerable lag between the formation of the storage vesicles and recovery of the catecholamine stores.

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[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

In the preceding paper (1) it was shown that normally filled, partially depleted, and fully depleted adrenal catecholamine storage vesicles can be differentiated by isopycnic centrifugation through sucrose density gradients. After reserpine treatment the catecholamine content is depleted but no dopamine β-hydroxylase (EC 1.14.21) is lost (1, 2). The storage vesicles from these animals, when centrifuged through a sucrose density gradient, equilibrate at a lower density and have a higher dopamine β-hydroxylase to catecholamine ratio than do vesicles obtained from adrenal glands of untreated animals (1). Although the storage vesicles secrete their soluble content by exocytosis during neurogenic stimulation (3–10), it has not previously been determined whether those vesicles which participate in the secretory response release all or only part of their content. The studies reported here provide evidence that neuronally induced secretion from the adrenal medulla, in contrast to reserpine-induced depletion, occurs by an "all-or-none" release of the contents of the storage vesicles. A preliminary report of this work has been published elsewhere (11).

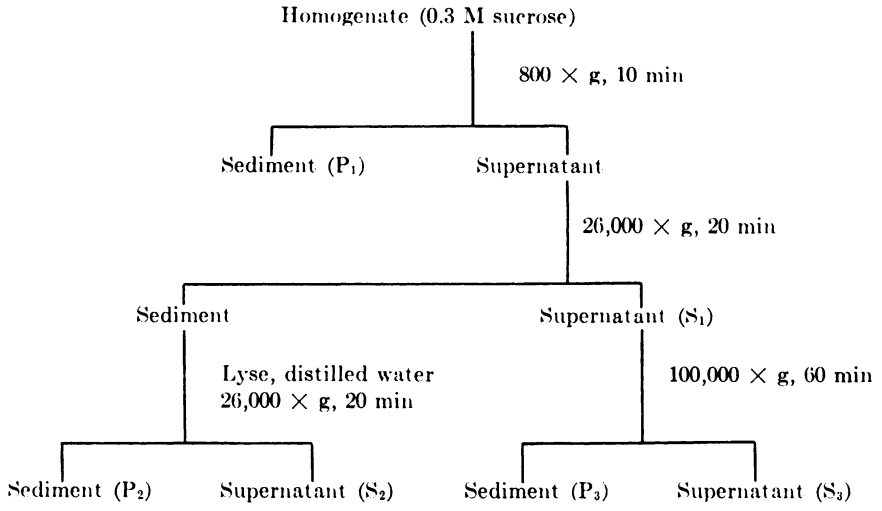
After neuronally induced depletion, 4–6 days are required for the rabbit adrenal gland to recover its catecholamine stores (12). During repletion, it is not known whether the storage vesicles are reused, whether entirely new vesicles must be assembled, or what the rate-limiting factors may be. Studies reported here of the recovery of the catecholamine stores following insulin-induced depletion suggest that synthesis of storage vesicles is not the rate-limiting step.

METHODS

Treatment of animals. All animals were a strain of New Zealand white rabbits and weighed 2–3 Kg. Animals which received insulin were fasted for 24 hr and then given 40 units of insulin per kilogram of body weight via the ear vein. Three or four hours after the insulin injection the animals either were killed or were brought out of hypoglycemic shock by administration of 15 ml of 50% sucrose by stomach tube and killed at various times afterward. If hypoglycemic symptoms reappeared, the administration of sucrose was repeated.

Preparation of homogenates for sucrose density centrifugation. Homogenates of adrenal glands were prepared and centrifuged through both light and heavy sucrose density gradients (1). Catecholamines and dopamine β-hydroxylase were assayed as described previously (13).

Differential centrifugation. Adrenal glands were obtained as described above, homogenized, and separated into subcellular frac-



SCHEME 1. *Differential centrifugation of adrenal homogenates*

tions as shown in Scheme 1. After centrifugation at 800 × *g* for 10 min, the particulate fraction (P_1) was rehomogenized in an amount of ice-cold distilled water equal to the volume of the original homogenate. The 800 × *g* supernatant fraction was centrifuged at 26,000 × *g* for 20 min. The supernatant fluid (S_1) was decanted and saved. The sediment was lysed by rehomogenization in distilled water to give a volume equal to the original homogenate and centrifuged at 26,000 × *g* for 20 min. The supernatant fraction (S_2) was saved for assay, and the sediment (P_2) was again suspended in the original volume of distilled water. The first 26,000 × *g* supernatant fraction (S_1) was centrifuged at 100,000 × *g* for 1 hr. The supernatant fluid (S_3) was decanted and saved for assay, and the sediment (P_3) was resuspended in water equal to the volume of the initial homogenate.

Materials. Insulin was obtained from Squibb and Sons, Inc. ^{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

Release of soluble dopamine β-hydroxylase and retention of particulate dopamine β-hydroxylase following insulin administration. Previous studies on the subcellular distribution of dopamine β-hydroxylase and catecholamines following various drug treatments indicated that only the soluble dopamine β-hydroxylase was released during secretion, and that the particulate fraction was retained by the gland (2). However, the total dopamine β-hydroxylase recovered in the crude storage vesicle fraction (26,000 × *g*) prepared from adrenal glands of insulin-treated animals was less than that expected if the pellet had contained all of the membranes from the disrupted vesicles as well as the remaining intact storage vesicles. To determine whether the apparent decrease in particulate dopamine β-hydroxylase might have been due either to some fragmentation of the vesicle membranes or to adhesion to other membranes resulting in particulate dopamine β-hydroxylase that did not sediment at 26,000 × *g*, more extensive fractionation studies than those previously reported were carried out.

Homogenates of adrenal glands prepared from control and insulin-treated animals were fractionated as shown in Scheme 1 and described in METHODS. The data in Table 1 show that the loss of dopamine β-hydroxylase 4 hr after insulin treatment occurred from the soluble fraction (S_2) of the storage vesicles. There was a decrease of the enzyme activity in the particulate fraction (P_2) of the storage vesicles, but this was compensated for by an equivalent increase of activity in the 100,000 × *g* pellet (P_3). Apparently some of the membranes of the storage vesicles had become fragmented to the extent that they no longer sedimented at 26,000 × *g*. Twenty-four hours after insulin treatment the dopamine β-hydroxylase activity in P_2 rose to normal levels and the activity in P_3 decreased to normal levels, but the activity in S_2 was still significantly below normal. The mean dopamine β-hydroxylase and catecholamine levels in P_2 and S_2 were greater at 24 hr than at 4 hr, suggesting that some resynthesis of storage vesicles and catecholamines had already occurred. The loss of dopamine β-hydroxylase activity in the P_3 fraction during the 4–24-hr interval may have been due either to reutilization of the particulate fraction for vesicle formation or to its destruction.

Because of the loss of catecholamines and soluble dopamine β-hydroxylase and the retention of all of the particulate dopamine β-hydroxylase, there was a significant increase in the ratio of total dopamine β-hydroxylase to total catecholamines at both 4 and 24 hr. However, in the S_2 fraction, from which catecholamines and dopamine β-hydroxylase were released, there was only a barely significant increase ($p < 0.05$) in the dopamine β-hydroxylase to catecholamine ratio at 4 hr, and by 24 hr this ratio was not different from the controls. These results confirm and extend our previous report that the soluble contents of storage vesicles are released to the exterior of the cell during secretion and that the storage vesicle membranes remain within the gland (2).

Effects of insulin treatment on storage vesicles. The crude storage vesicle fractions ob-

TABLE 1

Distribution of dopamine β-hydroxylase and catecholamines in subcellular fractions of adrenal homogenates

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

Treatment	Dopamine β-hydroxylase							
	P_1	P_2	S_2	P_3	S_3	Total	$P_1 + P_2 + P_3$	$S_2 + S_3$
	nmoles × 100/gland pr/hr							
None (7)[a]	161 ± 14	570 ± 54	703 ± 55	200 ± 28	276 ± 25	1840 ± 122	930 ± 70	979 ± 71
Insulin, 4 hr (5)	155 ± 23	361 ± 49[b]	202 ± 39[c]	462 ± 37[c]	208 ± 23[b]	1388 ± 23[b]	977 ± 95	410 ± 56[c]
Insulin, 24 hr (5)	125 ± 10[b]	464 ± 54	287 ± 41[c]	205 ± 25[c]	164 ± 21[d]	1240 ± 118[d]	794 ± 79	450 ± 53[c]
Treatment	Catecholamines							
	P_1	P_2	S_2	P_3	S_3	Total	$P_1 + P_2 + P_3$	$S_2 + S_3$
	μg/gland pr							
None (7)	6.6 ± 0.9	6.0 ± 1.0	58.7 ± 9.3	1.6 ± 0.4	10.7 ± 1.7	83.6 ± 12.9	12.5 ± 2.3	69.4 ± 10.7
Insulin, 4 hr (5)	1.4 ± 0.3[e]	1.1 ± 0.3[e]	10.8 ± 2.9[e]	0.8 ± 0.1	2.8 ± 0.7[f]	18.7 ± 4.7[e]	2.6 ± 0.4[d]	13.6 ± 3.5[e]
Insulin, 24 hr (5)	2.4 ± 0.5[f]	2.1 ± 0.3[d]	19.2 ± 4.1[d]	1.0 ± 0.2	3.6 ± 0.7[d]	28.2 ± 5.4[d]	5.6 ± 0.8[b]	22.8 ± 4.8[d]
Treatment	Dopamine β-hydroxylase to catecholamine ratio							
	P_1	P_2	S_2	P_3	S_3	Total	$P_1 + P_2 + P_3$	$S_2 + S_3$
	nmoles × 100/hr/μg							
None (7)	26.2 ± 2.8	103 ± 11	14 ± 1.9	153 ± 24	29 ± 4.3	24 ± 2.9	84 ± 10.7	16 ± 2.2
Insulin, 4 hr (5)	122 ± 17[c]	436 ± 106[d]	20 ± 1.7[b]	621 ± 52[c]	89 ± 17[g]	93 ± 19[e]	406 ± 52[c]	35 ± 4.4[f]
Insulin, 24 hr (5)	58 ± 9[c]	247 ± 46[d]	16 ± 1.3	237 ± 40	53 ± 13	50 ± 9[g]	155 ± 24[g]	22 ± 3.3

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

tained from adrenal glands of control and insulin-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 (Fig. 1). The data were grouped into five segments of the gradient as previously described (1). 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 into 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 position of the peaks of dopamine β-hydroxylase and catecholamines in the gradients, the number of fractions from the denser 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.

Three hours after insulin treatment there was a decrease in the total content of dopamine β-hydroxylase and catecholamines but no change in the position of the peaks. The catecholamine content was lowered in all segments of the gradients prepared from vesicles of insulin-treated animals, and the dopamine β-hydroxylase content was likewise reduced, except in fraction D. From Table 2 it can be seen that the percentage loss of dopamine β-hydroxylase from the storage vesicles (A and B) was approximately the same as that of catecholamines. The dopamine-β-hydroxylase to catecholamine ratio of segment A was only slightly less than that of the controls, and there was no difference in the dopamine β-hydroxylase to catecholamine ratios in segment B. In segments C and D of the gradient, as well as in the total fraction applied to the gradient, the dopamine β-hydroxylase to catecholamine ratios of the insulin-treated group were greater than those of the controls. The total dopamine β-hydroxylase activity in fraction

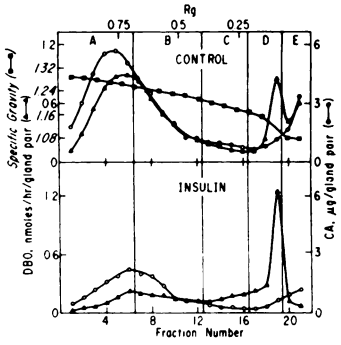


FIG. 1. *Isopycnic centrifugation, through "heavy" sucrose density gradients, of crude storage vesicle fraction obtained from control and insulin-treated animals*

DBO, dopamine β-hydroxylase; CA, catecholamines.

D of control animals was approximately 10% of the total activity applied to the gradient, and probably arose from membranes of vesicles disrupted during the preparation. In insulin-treated animals the dopamine β-hydroxylase activity in fraction D was approximately 45% of the total activity applied to the gradient. Part of the activity in this fraction may have been derived from membranes of vesicles disrupted during preparation of the sample, and part from membranes of vesicles disrupted during secretion. Thus treatment of rabbits with insulin caused depletion of both the dopamine β-hydroxylase and the catecholamine content of the storage vesicles. This treatment produced a relative increase in dopamine β-hydroxylase in segment D, where membranes of lysed vesicles equilibrate (1), but did not change the relative amounts of dopamine β-hydroxylase and catecholamines in the storage vesicles, nor did it affect their buoyant density.

Twenty-four hours after insulin treatment there were no apparent differences in the total catecholamine content and dopamine β-hydroxylase activity compared to the levels observed 3 hr after insulin treatment. However, there were significant changes in the distribution of dopamine β-hydroxylase on the density gradients. In the 3-hr group, segments B and D contained 21 and 46% of the total dopamine β-hydroxylase activity,

TABLE 2

Effect of insulin on distribution of dopamine β-hydroxylase and catecholamines in sucrose density gradients

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

Treatment	Dopamine β-hydroxylase						
	A	B	C	D	E	Total	R_g
	nmoles × 100/gland pr/hr						
None (7)[a]	412 ± 46	441 ± 46	66 ± 2	126 ± 8	90 ± 8	1130 ± 89	0.74 ± 0.07
Insulin, 3 hr (6)	43 ± 6[b]	73 ± 11[b]	48 ± 7[c]	159 ± 26	25 ± 5[b]	345 ± 50[b]	0.72 ± 0.02
Insulin, 24 hr (5)	40 ± 3[b]	128 ± 11[b]	61 ± 5	54 ± 2[b]	28 ± 3[b]	312 ± 19[b]	0.53 ± 0.05
Insulin, 48 hr (4)	244 ± 24[d]	501 ± 9	108 ± 10[b]	105 ± 6	57 ± 17	1016 ± 49	0.70 ± 0.02
Insulin, 96–144 hr (5)	434 ± 74	462 ± 73	97 ± 18	97 ± 11	96 ± 21	1186 ± 15	0.76 ± 0.01
Treatment	Catecholamines						
	A	B	C	D	E	Total	R_g
	μg/gland pr						
None (7)	32.1 ± 4.0	25.9 ± 3.4	5.5 ± 0.9	4.0 ± 0.4	6.7 ± 0.8	74.3 ± 9.0	0.79 ± 0.01
Insulin, 3 hr (6)	5.4 ± 0.6[b]	4.5 ± 0.6[b]	1.0 ± 0.1[b]	0.8 ± 0.1[b]	1.6 ± 0.2[b]	13.3 ± 1.3[b]	0.77 ± 0.02
Insulin, 24 hr (5)	3.7 ± 0.6[b]	6.7 ± 1.0[b]	3.5 ± 0.9	1.7 ± 0.2[e]	1.5 ± 0.2[b]	17.2 ± 2.0[b]	0.60 ± 0.05[e]
Insulin, 48 hr (4)	7.6 ± 1.5[e]	11.0 ± 2.8[f]	3.5 ± 0.8	1.0 ± 0.3[d]	2.4 ± 0.3[g]	16.5 ± 5.0[g]	0.75 ± 0.02
Insulin, 96–144 hr (5)	29.0 ± 5.1	19.6 ± 2.8	5.1 ± 0.6	4.1 ± 0.7	5.6 ± 0.7	63.8 ± 8.3	0.77 ± 0.02
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	
Insulin, 3 hr (6)	8.1 ± 0.8[c]	16.4 ± 2.1	49.7 ± 8.7[b]	190 ± 31[b]	16.2 ± 3.3	25.9 ± 3.6[f]	
Insulin, 24 hr (5)	12.0 ± 2.1	21.5 ± 4.6	21.0 ± 4.4	34.8 ± 5.8	19.0 ± 2.2	19.6 ± 3.2	
Insulin, 48 hr (4)	35.0 ± 5.7[e]	55.8 ± 14.6[d]	35.7 ± 7.7[d]	53.4 ± 4.4[e]	22.0 ± 3.7[e]	42.3 ± 7.4[e]	
Insulin, 96–144 hr (5)	16.3 ± 3.8	24.2 ± 3.6	18.9 ± 2.6	26.7 ± 5.1	19.2 ± 5.0	19.3 ± 3.0	

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

respectively, compared to 39 and 11% for the control group and 41 and 17% for the 24-hr group. Hence the distribution of dopamine β-hydroxylase in the 24-hr group was similar to that of the control group, even though the 24-hr post-insulin group was markedly depleted of both dopamine β-hydroxylase and catecholamines. The redistribution was due largely to a loss of activity in segment D and an increase in activity in segment B. The distribution of activities at 24 hr was not entirely the same as that of the untreated animals; in the controls the dopamine β-hydroxylase and catecholamine contents were distributed approximately equally between segments A and B, but 24 hr after insulin treatment segment B contained about twice the amount of catecholamines and 3 times the amount of enzyme activity as segment A. The dopamine β-hydroxylase to catecholamine ratios of the vesicles in all segments of the gradients at 24 hr were not significantly different from the control groups, but the vesicles had a lower buoyant density. In contrast, the vesicles from reserpine-treated groups at 24 hr had a lower buoyant density than the controls, but the dopamine β-hydroxylase to catecholamine ratios were considerably higher (1).

There was a dramatic increase in the dopamine β-hydroxylase activity between 24 and 48 hr. At 48 hr the dopamine β-hydroxylase levels had increased to normal limits, but the catecholamine content was only 35% of the controls. The vesicles in segments A and B had the same modal buoyant density as those of the control animals, but the dopamine β-hydroxylase to catecholamine ratios were significantly greater and resembled those of reserpine-treated animals (1). At the same time the dopamine β-hydroxylase activity in segment B was the same as that of the controls but the activity in segment A was only 60% of the control levels. The distribution of both dopamine β-hydroxylase and catecholamines at 48 hr was similar to that at 24 hr, but there was a significant increase in the dopamine β-hydroxylase activity in segment C. These observations suggest that vesicles which have recovered only a portion of their catecholamine content have a lower buoyant density on sucrose gradients than do normal vesicles and that there is a significant delay between the synthesis of new vesicles, or the repair of the old vesicles, and repletion of the catecholamine stores. The vesicles examined from 96 to 144 hr after insulin treatment did not differ from the controls in any of the measured parameters.

Distribution of particulate and soluble dopamine β-hydroxylase and catecholamines in heavy sucrose density gradients. The distribution of dopamine β-hydroxylase and catecholamines in dense sucrose gradients suggested that the bulk of the dopamine β-hydroxylase and catecholamines present in the 26,000 × *g* sediment (crude vesicle fraction) equilibrate in a well-defined peak near the bottom of the gradient, and that a second peak of dopamine β-hydroxylase activity occurs near the top of the gradient. The distribution of bound and readily soluble enzyme in these fractions had not previously been determined, and it was therefore of interest to carry out this analysis.

The 26,000 × *g* sediments containing the storage vesicles from control animals and from animals 3 hr after insulin treatment were resuspended in 0.3 M sucrose and centrifuged through a dense sucrose gradient as described in METHODS. Two milliliters of ice-cold water were added to each of the fractions obtained from the gradient, and the suspensions were frozen and thawed twice to lyse the vesicles. The fractions were then centrifuged at 26,000 × *g* for 20 min, and the soluble and particulate fractions were assayed for both dopamine β-hydroxylase and catecholamines. Figure 2 and Table 3

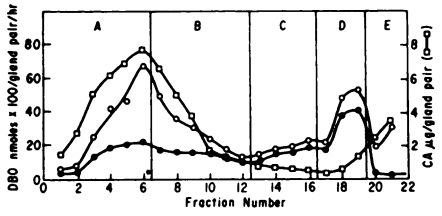


FIG. 2. *Distribution on "heavy" sucrose density gradients of catecholamines (CA), total dopamine β-hydroxylase (DBO, ○——○), and dopamine β-hydroxylase present in water-insoluble residue after lysis (●——●)*

The crude storage vesicle fraction was prepared from adrenal glands of untreated rabbits.

TABLE 3

Distribution of particulate dopamine β-hydroxylase in sucrose density gradients

For details of the procedures, see corresponding sections of RESULTS and METHODS. The data were obtained in a single experiment. A duplicate experiment gave essentially the same results.

Treatment	Dopamine β-hydroxylase					
	A	B	C	D	E	Total
	% total					
Control	41	50	82	78	11	53
Insulin, 3 hr	52	57	85	85	86	74

give the results of these experiments. The distribution of particulate and soluble dopamine β-hydroxylase in gradients prepared from vesicles of insulin-treated animals was essentially the same as that of control animals. In fractions 2–10, which contained the bulk of the catecholamines and dopamine β-hydroxylase applied to the gradient, the particulate dopamine β-hydroxylase obtained from controls was 42% of the total enzyme activity in these fractions and the particulate dopamine β-hydroxylase of the insulin-treated animals was 52% of the total. Most of the dopamine β-hydroxylase in fractions C and D was particulate. Essentially all of the catecholamines were recovered in the soluble fractions. The high percentage of particulate dopamine β-hydroxylase in segment E from insulin-treated animals was probably due to contamination with some particulate dopamine β-hydroxylase from the preceding fraction.

Buoyant density of 100,000 × g particulate dopamine β-hydroxylase. The buoyant density of dopamine β-hydroxylase in the 100,000 × *g* sediment was determined by centrifugation through "light" sucrose density gradients (1). Figure 3 shows the distribution of catecholamines and dopamine β-hydroxylase in the 26,000 × *g* fractions obtained from control animals and from animals 3 hr after treatment with insulin. The buoyant density of the dopamine β-hydroxylase activity in the 100,000 × *g* sediment obtained from the glands of insulin-treated animals is the same as that of the minor peaks of dopamine β-hydroxylase found after centrifugation of the 26,000 × *g* intact vesicles, and corresponds to the activity in segment D of the "heavy" sucrose density gradients.

DISCUSSION

There is an excess of soluble dopamine β-hydroxylase in the S_3 fraction (Table 1), which apparently cannot be accounted for by breakage of the storage vesicles during homogenization of the gland. If the assumption is made that in control animals there is a uniform population of vesicles, and that all, or nearly all, of the catecholamines are present in the vesicles, then the fraction of the total soluble catecholamines present in S_3, i.e., $S_3/(S_2 + S_3)$, should represent the fraction of vesicles disrupted during the preparative procedure. In the untreated animals this represented approximately 15% of the total vesicle population. Hence 15% of the total soluble dopamine β-hydroxylase ($S_2 + S_3$), equal to 150 units of enzyme activity, should also have been present in the S_3 fraction. However, the S_3 fraction contained 276 units, leaving 126 units unaccounted for. Similar calculations for the groups that had received insulin 4 and 24 hr earlier showed excess amounts of 126 and 89 units, respectively. The excess dopamine β-hydroxylase may be normally present in the soluble cytoplasm, or, as seems more likely, it may come from a population of vesicles which have higher dopamine β-hydroxylase to catecholamine ratios than the

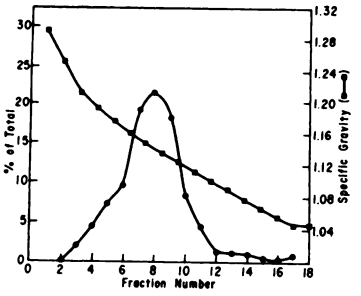


FIG. 3. *Isopycnic centrifugation of 100,000 × g sediment through "light" sucrose density gradients*

The distribution of dopamine β-hydroxylase present in the 100,000 × *g* sediment prepared from adrenal glands of untreated rabbits is shown.

average. The data of Table 2 show that storage vesicles obtained from animals 48 hr after insulin treatment had dopamine β-hydroxylase to catecholamine ratios 3 times greater than vesicles obtained from untreated animals. This suggests that the excess enzyme present in S_3 arose from newly formed or newly forming vesicles which had not yet acquired their full catecholamine content.

Studies from this and other laboratories have provided evidence that secretion from the adrenal medulla occurs by exocytosis, a process by which the soluble contents of the storage vesicles are secreted directly to the exterior of the cell (3–10), leaving the vesicle membranes within the cell (2). However, these studies did not indicate whether the secreted products came from a population of storage vesicles which released a portion of their contents or from a smaller population of the same vesicles which released their total contents. The data in Table 2 and Fig. 1 provide evidence that each of the vesicles which secretes releases its total content in an apparent "all-or-none" response. These observations suggest that secretion from the adrenal medulla is quantal and that the storage vesicles are the subcellular quantal units. This does not imply that each cell of the gland will respond to the same stimulus by releasing an equal number of quanta, or that the same cell will respond to successive equal stimuli by releasing the same number of quanta each time. By quantal secretion we mean that the minimal secretory response of a chromaffin cell to a stimulus is the release of the total content of one storage vesicle, and that any further response will consist of the release of the total content of an integral number of storage vesicles.

The experimental conditions do not rule out the possibility that total release of the vesicle content occurred, not in response to a single stimulus, but as a result of repeated responses to successive stimuli. For example, the vesicle may release only a portion of its content in response to the first stimulus, but because of a favored proximity or apposition to the cell membrane, it may respond to subsequent stimuli until completely depleted. Our data on the dopamine β-hydroxylase to catecholamine ratios of the purified storage vesicles do not support this interpretation, and electron microscopic studies do not indicate an increased number of vesicles attached to the cell membranes of stimulated glands (14–16). However, the electron microscope studies were carried out on glands which were fixed after removal from the animals, and such attachments may have been lost during this time. Fixation of the tissue by perfusion with glutaraldehyde *in vivo*, as described by Diner (17), may provide information relevant to this point.

The depletion of adrenal catecholamines by reserpine in the presence of ganglionic blocking agents differs from that caused by neurogenic stimulation. Reserpine treatment following ganglionic blockade results in depletion of catecholamines, no loss of either soluble or particulate dopamine β-hydroxylase, and a decrease in the buoyant density of the vesicles (1). These observations are consistent with the direct effects of reserpine on the storage vesicles. Depletion of catecholamines occurs because reserpine blocks both the ability to reaccumulate epinephrine and norepinephrine which may have leaked into the cytoplasm from the storage vesicles, and the ability to take up dopamine into the storage vesicles, where it is oxidized to norepinephrine.

There is little information on the events which occur upon cessation of stimulation. The data of Table 1 and Figs. 1 and 3 suggest that either during secretion or shortly thereafter the membranes of the "empty" storage vesicles are cleaved to smaller fragments without loss of particulate dopamine β-hydroxylase. Neurosecretory granules of the blowfly, *Calliphora erythrocephala* (18), zymogen granules of the salivary gland (19), and neurosecretory granules of the posterior pituitary gland (20) have been reported to form smaller vesicles following secretion. Electron micrographs of the adrenal glands of hamsters (17) also suggest that the storage vesicles, after release of their contents, form smaller vesicles. The finding that there was no increase in dopamine β-hydroxylase activity in the 800 × *g* pellet (Table 1) might indicate that fusion or adhesion of the storage vesicles to the

plasma membrane is only short-lived in the adrenal medulla compared to the salivary gland (19), and may explain the difficulty in observing this phenomenon by electron microscopy. Poisner *et al.* (21) and Malamed *et al.* (22) arrived at similar conclusions from their studies of lipid distribution in homogenates of bovine adrenal medullae after stimulation of the isolated glands with acetylcholine.

Twenty to twenty-one hours after stimulation was stopped, the dopamine β-hydroxylase content of the glands was redistributed compared to those examined 3 or 4 hr after administration of insulin. Although there was no increase in either total dopamine β-hydroxylase or total catecholamines in the 3–24-hr period, the decreased content of enzyme in fraction P_3 (Table 1) and segment D (Table 2) and the increase in fraction P_2 and segment B suggest that some recovery of intact storage vesicles had occurred. The reductions in enzyme activity in P_3 and segment D are at least twice as large as the increases in fraction P_2 and segment B, respectively, suggesting that at least some of the old membranes were destroyed and not reutilized for the formation of new vesicles.

The formation of new vesicles appeared to be virtually complete 45 hr after secretion had stopped, but at this time the vesicles did not contain their normal complement of catecholamines even though the dopamine β-hydroxylase levels were the same as in vesicles from control animals. Although the R_g value was the same as that of the controls, the distribution of catecholamines and dopamine β-hydroxylase in segments A and B 48 hr after insulin was much more asymmetrical and displaced toward the less dense portion of the gradient. The low levels of catecholamines probably were not due to a low synthetic capability, since at this time the tyrosine hydroxylase activity was twice that of control animals (12) and the dopamine β-hydroxylase activity was within normal limits. Under normal conditions the gland has sufficient dopamine β-hydroxylase activity to synthesize its total content of epinephrine and norepinephrine within 15 min (13), and sufficient tyrosine hydroxylase to synthesize an equivalent amount of dopa in 21–22 hr when the enzymes are assayed under nearly optimal conditions *in vitro* (12). However, the activities of either or both of these enzymes may be tightly regulated in the intact cell, and one cannot extrapolate optimal activities measured *in vitro* to activities *in vivo*. A second factor which may be of importance in the recovery of the catecholamine content is the ability of the storage vesicles to take up and store the amine. Previous studies have shown that neurogenic stimulation results in a decreased ability of the isolated storage vesicles to take up exogenous amines, and that there is a parallel recovery of catecholamine stores and the ability to take up exogenous amines (12). The data of Table 2 and other studies (12) suggest that formation of new storage vesicles is not per se the rate-limiting step in the recovery of the catecholamine stores, but that biosynthesis of the amine or development of the uptake and storage process may be rate-limiting.

In the past, the adrenal medulla has been a useful model for studies of the sympathetic nerves. The enzymes for the synthesis of norepinephrine and their distribution appear to be identical in both tissues (23–25). Both tissues have storage mechanisms which appear to be similar but not identical (23, 25), and recent evidence suggests that chromogranins and dopamine β-hydroxylase are released from sympathetic nerve endings during stimulation (25–28), as has been observed in the adrenal medulla (5–10). Whether release of norepinephrine at sympathetic nerve endings is quantized in the sense described here has yet to be demonstrated. Folkow *et al.* (29) and Stjärne *et al.* (30) have argued against the hypothesis that the synaptic vesicle is the subcellular quantal unit. They have calculated that only 3–10% of the content of a storage vesicle per varicosity is released for each maximal stimulus delivered to the vasculature of striated muscle. Their arguments are compelling but are not supported by sufficient direct evidence to exclude either exocytosis as the mechanism of secretion at nerve endings, or the synaptic vesicle as the subcellular quantal unit. It should be noted that neither Folkow *et al.* (29) nor Stjärne *et al.* (30) argued against the concept of quantal release at sympathetic nerve endings (31–33), but

addressed themselves to the size and site of the quantal unit. Calculations for the adrenal medulla indicate that the amount of catecholamines released per maximal stimulus applied to the splanchnic nerve can be accounted for by the release of the total content of one vesicle per cell, assuming a uniform response by all the cells of the gland (34).

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