Mechanism of Secretion from the Adrenal Medulla
V. Retention of Storage Vesicle Membranes following Release of Adrenaline

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

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

(Received February 1, 1969)

SUMMARY

Dopamine β-hydroxylase (EC 1.14.2.1) was used as a marker to follow the fate of the adrenal storage vesicles following the release of adrenaline. Neurogenic secretion of adrenaline induced by insulin produced a decrease in the adrenaline and dopamine β-hydroxylase contents of the gland. There was only a slight decrease in the enzyme activity of the particulate fraction obtained from lysed storage vesicles, but a marked decrease in the activity of the soluble fraction. Twenty-four hours after the administration of reserpine (1 mg/kg) to rabbits, changes in the dopamine β-hydroxylase activity were similar to those observed after insulin treatment, but the glands were more severely depleted of their catecholamines. After lower doses of reserpine (0.25 mg/kg), or if ganglionic transmission was blocked by chlorisondamine 1 hr prior to the administration of reserpine (1 mg/kg), there were significant decreases in the catecholamine content but no changes in the dopamine β-hydroxylase activity. These findings indicate that neurogenic secretion is characterized by release of the soluble content of the storage vesicles directly to the exterior of the cell and retention of the storage vesicle membranes within the cell. Interference only with the uptake or storage mechanism by reserpine results in depletion of the catecholamine content, but not of the intravesicular protein.

INTRODUCTION

During secretion of catecholamines from the adrenal medulla, the soluble contents of the storage vesicles are released directly to the exterior of the cell (1–7). Electron microscopic (8–10), chemical (11, 12), and enzymatic (13) studies suggest that the vesicle membranes remain within the cell after secretion. The fact that a portion of the dopamine β-hydroxylase (EC 1.14.2.1) activity of the adrenal gland is firmly attached to the vesicle membranes (14, 15) makes it possible to use this enzyme as a marker to follow the fate of these membranes after the release of catecholamines. The studies reported here show that the dopamine β-hydroxylase associated with the particulate fraction of the storage vesicles is largely retained within the cell following secretion of catecholamines.

This work was supported by grants from the National Science Foundation (GB 5812), the National Institutes of Health (AM 05427), and the American Medical Association Education and Research Foundation.

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

[2] Research Career Awardee (K3-GM-15814) of the National Institutes of Health.

[3] O. H. Viveros, unpublished observations.

METHODS

Treatment of animals. All animals were a strain of New Zealand white rabbits weighing 2–3 kg. Animals receiving insulin were fasted for 24 hr and then given 40 units of insulin per kilogram of body weight via the ear vein. Three hours after the injection of insulin, the animals either were killed or were brought out of insulin shock by administration of 5 ml of 50% sucrose

by stomach tube and killed 21 hr later. Animals which received chlorisondamine chloride, reserpine, or a combination of the two drugs were not fasted. Reserpine was administered intravenously via the ear vein; chlorisondamine was injected intraperitoneally. Animals which received both reserpine and chlorisondamine were treated with the latter 1 hr before administration of reserpine. The reserpine-treated animals were killed 12 or 24 hr later, as indicated. For each series of assays, one untreated animal and two or three treated animals were used. Chlorisondamine (10 mg/kg) was used to block neurogenic stimulation of the adrenal gland.

Preparation of homogenates. The rabbits were killed by a blow on the base of the skull, and their 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 homogenates were centrifuged at 26,000 $\times$ *g* for 20 min at 5°, and the supernatant fraction (S_1) was decanted. The particulate fraction (P_1) was homogenized in ice-cold water to lyse the storage vesicles, in order to give a mixture equivalent to 50 mg of original tissue per milliliter. The mixture was centrifuged at 26,000 $\times$ *g* for 20 min, and the supernatant fraction (S_2) was decanted. The particulate fraction (P_2) was finally suspended in ice-cold water to give a mixture equivalent to 50 mg of original material per milliliter. The fractionation procedure is shown diagrammatically in Fig. 1. Aliquots of S_1 and P_1 were assayed for catecholamines as described previously (13). Aliquots of S_1, P_2, and S_2 were assayed for dopamine β-hydroxylase. During the early part of this work, aliquots of P_1 were also assayed for dopamine β-hydroxylase, but in 25 pairs of glands, all of the activity in P_1 could be accounted for by the activities found in S_2 and P_2. The values for P_1 in Table 1 are the sum of P_2 plus S_2.

Assay of dopamine β-hydroxylase. Each of the fractions was assayed in the presence of two different concentrations of *p*-hydroxymercuribenzoate to inactivate the endogenous inhibitors (14). For the assay of S_1, the reaction mixtures contained 2 or 5×10^{-4} M mercuribenzoate; for the assay of P_1, P_2, and S_2, the mixture contained 1 or 2×10^{-4} M mercuribenzoate. Generally the activities measured in the two mixtures differed by less than 10%, but in all cases the highest measured activity was recorded. In addition to *p*-hydroxymercuribenzoate, the reaction mixtures contained potassium phosphate buffer (pH 6.0), 100 mM; sodium fumarate (pH 6.0), 10 mM; sodium ascorbate (pH 6.0), 1.0 mM; ATP (pH 6.0), 5 mM; tranylcypromine, 0.5 mM; catalase, 400 units; ^{3}H-tyramine (generally labeled, 100 μCi/μmole), 0.01 mM; and 0.2 ml of the tissue fraction in a total volume of 1.0 ml. The reaction mixtures were incubated at 37° for 15 min in air. Under these conditions the assay was approximately linear with time for at least 15 min, and was linear with the amount of tissue added within the limit used here (15). The reaction was stopped by the addition of 1.0 ml of 7%

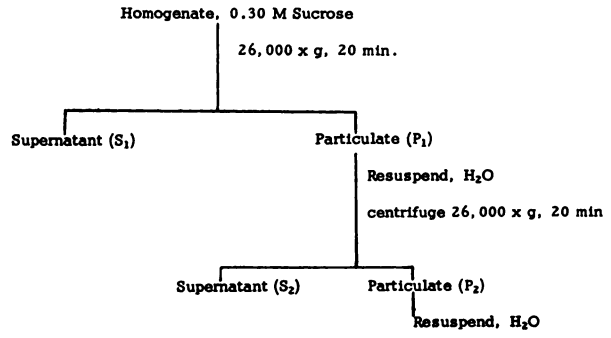

FIG. 1. *Schematic presentation of the procedure used to prepare subcellular fractions of adrenal glands*

perchloric acid. After removal of the precipitate, a 1-ml aliquot was assayed for the amount of ^{3}H-octopamine formed by the periodate oxidation method of Friedman and Kaufman (16).

Statistical methods. Standard errors of the mean were calculated from the formula

$$SE = \frac{X^2 - N(X/N)^2}{N\sqrt{N-1}}$$

Student's *t*-test was used to determine statistical significance.

Materials. Insulin was obtained from Squibb and Sons, Inc., and reserpine (Serpasil) and chlorisondamine chloride (SU 3088), 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 absorption on a column (0.5 × 3 cm) of Dowex 50-H$^+$, followed by elution with 0.5 N HCl.

RESULTS

Seven groups of animals were used in the experiments reported here: one control group and six other groups, each receiving a different treatment as indicated in Table 1. Of major interest to the following discussion are the catecholamine contents of the different groups and the dopamine β-hydroxylase activities in fractions P_2 and S_2. The results were calculated in terms of mμmoles of octopamine formed per hr per gland pair and as mμmoles of octopamine formed per hr per 100 mg of gland. Only minor differences in the results were found between both forms of expression. The reported *p* values are for the gland pair calculations. There were no significant differences in the mean weights of the gland pairs among the seven groups.

In comparison with the control group, there was a significant decrease in the adrenal catecholamine content of each of the drug-treated groups. The most marked effect was observed in the group assayed 24 hr after receiving reserpine (1 mg/kg). The decrease in the chlorisondamine-treated group was only barely significant when expressed on the basis of gland pairs (Table 1), but not significant when calculated per 100 mg of gland.

Twenty-four hours after the administration of reserpine (1 mg/kg), and 3 and 24 hr after the administration of insulin, there were significant decreases in the total dopamine β-hydroxylase activities. These decreases were due almost entirely to losses in the activities of the P_1 fractions. There were no differences in the S_1 fractions except in the group treated with 0.25 mg of reserpine, which showed an increase in activity. The decreases in activity of the P_1 fraction were due mainly to the loss of enzyme activity from the S_2 fraction. The activity in the S_2 fraction had decreased to 19 ± 2%, 33 ± 4%, and 53 ± 9% of the control values, respectively, for the animals assayed 24 hr after receiving 1 mg of reserpine per kilogram and 3 and 24 hr after receiving insulin, but the dopamine β-hydroxylase activities in the P_2 fractions had declined to only 67 ± 6%, 63 ± 6%, and 77 ± 6%, respectively, of the control group. In the reserpine-treated group and in the group assayed 3 hr after insulin injection, the relative decrease of enzyme activity in the S_2 fraction was 2–3 times that of the P_2 fraction. The decrease of activity in the P_2 fractions of these two groups was only barely significant, but the decrease in activity of the S_2 fraction was highly significant. The groups that received chlorisondamine, chlorisondamine + reserpine (1 mg/kg), or reserpine (0.25 mg/kg) alone showed no differences from the controls or among themselves in the enzyme activities of the P_1, P_2, and S_2 fractions.

Chlorisondamine itself had no effect on the dopamine β-hydroxylase activities of the subcellular fractions. When given 1 hr prior to the administration of reserpine (1 mg/kg), it only partially inhibited the depletion of catecholamines and completely blocked the decrease in enzyme activities observed after treatment with reserpine (1 mg/kg) alone. The adrenal catecholamine content of the group which received chlorisondamine plus reserpine was the same as that of the group assayed 3 hr after

TABLE 1

Effect of various treatments on the dopamine β-hydroxylase activity and adrenaline content of rabbit adrenal glands

The drugs were administered from 3 to 24 hr, as indicated, prior to killing the animals. The values are the means ± standard error for the indicated N. S_1, P_1, P_2, and S_2 are described in METHODS. All p values are in reference to the untreated group.

Treatment	N	Dopamine β-hydroxylase activity					Adrenaline per gland pair	Gland weight pair
		Total	S_1	P_1	P_2	S_2		
		mμmoles octopamine formed/hr/gland pair					*μg*	*mg*
None	7	14.2 ± 1.0	4.1 ± 0.5	10.9 ± 1.1	5.2 ± 0.7	5.7 ± 0.7	122 ± 11	203 ± 23
Chlorisondamine, 10 mg/kg, 24 hr	5	19.1 ± 0.6[a]	5.4 ± 0.6	13.7 ± 0.4	6.2 ± 1.1	7.6 ± 0.9	85 ± 7[b]	171 ± 21
Chlorisondamine, 10 mg/kg, + reserpine, 1 mg/kg, 24 hr	5	20.2 ± 3.2	4.9 ± 0.9	15.3 ± 2.4	5.4 ± 0.5	9.9 ± 2.0	42 ± 5[c]	198 ± 16
Reserpine, 1 mg/kg, 24 hr	7	7.9 ± 0.7[c]	3.1 ± 0.4	4.6 ± 0.4[c]	3.5 ± 0.3[b]	1.1 ± 0.1[c]	2.3 ± 0.5[c]	233 ± 20
Reserpine, 0.25 mg/kg, 12 hr	5	21.8 ± 3.7	8.3 ± 1.3[d]	13.5 ± 2.8	4.5 ± 0.9	9.0 ± 2.3	62 ± 17[d]	264 ± 30
Insulin, 40 units/kg, 3 hr	7	8.3 ± 1.0[e]	3.3 ± 0.4	5.2 ± 0.5[c]	3.3 ± 0.3[b]	1.9 ± 0.2[c]	36 ± 6[c]	163 ± 18
Insulin, 40 units/kg, 24 hr	7	10.5 ± 1.1[b]	3.4 ± 0.5	7.0 ± 0.7[f]	4.0 ± 0.3	3.0 ± 0.5[d]	45 ± 7[c]	199 ± 34

[a] $p < 0.005$.
[b] $p < 0.05$.
[c] $p < 0.001$.
[d] $p < 0.01$.
[e] $p < 0.002$.
[f] $p < 0.02$.

receiving insulin. However, the enzyme content of the insulin-treated group was significantly lower than that of the group given chlorisondamine plus reserpine.

Part of the results of Table 1 are displayed in Fig. 2 as a percentage of the control values. Although there were significant changes in the absolute amounts of catecholamines and dopamine β-hydroxylase in some of the drug-treated groups (Table 1), there were no shifts in the distribution between the S_1 and P_1 fractions (Fig. 2A and B). The P_1 fraction of the untreated group contained $82 \pm 2\%$ of the total catecholamines and $71 \pm 2\%$ of the total dopamine β-hydroxylase. There were, however, significant decreases in the ratio of dopamine β-hydroxylase activity in the S_2 and P_1 fractions (Fig. 2C) of the groups assayed after treatment with reserpine (1 mg/kg) and 3 and 24 hr after insulin. There were also significant decreases in the distribution of dopamine β-hydroxylase between the S_2 and P_2 fractions (Fig. 2D) in the groups assayed 3 hr after insulin treatment and 24 hr after receiving reserpine (1 mg/kg). In contrast, the ratios of dopamine β-hydroxylase activity in the S_2 and P_2 fractions of the groups which received chlorisondamine, chlorisondamine + reserpine (1 mg/kg), and reserpine (0.25 mg/kg) alone were higher than the control values, but only the ratio in the last-named group was significantly greater than that of the controls.

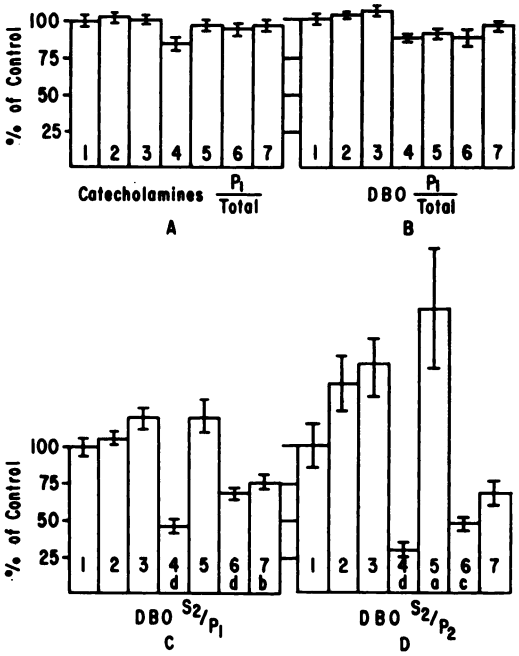

FIG. 2. *Ratios of catecholamines and dopamine β-hydroxylase (DBO) in various subcellular fractions, expressed as a percentage of the untreated group*

The vertical lines in the center of each bar are the standard errors of the means. The numbers within each bar refer to the following: *1*, no treatment; *2*, chlorisondamine, 10 mg/kg, 24 hr prior to assay; *3*, chlorisondamine, 10 mg/kg, plus reserpine, 1 mg/kg, 24 hr prior to assay; *4*, reserpine, 1 mg/kg, 24 hr prior to assay; *5*, reserpine, 0.25 mg/kg, 12 hr prior to assay; *6*, insulin, 40 units/kg, 3 hr prior to assay; *7*, insulin, 40 units/kg, sucrose administered 3 hr later, animals assayed 24 hr after insulin administration. The letters within the bars refer to the following p values: *a*, $p < 0.05$; *b*, $p < 0.01$; *c*, $p < 0.005$; *d*, $p < 0.001$.

DISCUSSION

Three operationally defined pools of dopamine β-hydroxylase were found in homogenates of rabbit adrenal glands prepared in 0.3 M sucrose. Approximately 30% of the activity was found in the supernatant fraction obtained after centrifuging the homogenates for 20 min at $26{,}000 \times g$. The activity in the particulate fraction is associated with the storage vesicles (15, 17), and approximately 50% is readily solubilized upon extraction with distilled water. The remainder is attached to the membranes of the storage vesicles and may be used as a membrane marker.

It has been shown that during acetylcholine-induced secretion from isolated, perfused bovine adrenal glands, dopamine β-hydroxylase and catecholamines are secreted in the same relative amounts as found in the water-soluble fraction of storage vesicles lysed with distilled water (18). It was also reported that after insulin-induced adrenal secretion in rabbits there was a decrease in the total dopamine β-hydroxylase activity, largely accounted for by a decrease of the enzyme in the $26{,}000 \times g$ particulate fraction (13). The studies reported here confirm these results and also show that the decrease in activity of the

particulate fraction was due mostly to a loss of the water-soluble enzyme.

There was a small decrease of activity in the P_2 fractions of the animals assayed 3 hr after insulin and 24 hr after reserpine treatment (1 mg/kg), but no apparent change in the S_1 fractions. A further analysis[3] has been carried out on the S_1 fraction of untreated and insulin-treated animals by centrifugation at 100,000 $\times$ g for 1 hr and measurement of the dopamine β-hydroxylase activities in the pellet and supernatant fractions. These studies have shown an increase of enzyme activity in the 100,000 $\times$ g pellet and a decrease in the supernatant fraction of the insulin-treated animals, such that the sum of the enzyme activities in the P_2 fraction and in the 100,000 $\times$ g pellet obtained from the insulin-treated animals was equal to the sum of these activities in the untreated animals. Thus there appears to be no loss of the particulate fraction during secretion. However, these observations do not exclude the possibility that a portion of the membrane-bound enzyme may have been discharged or inactivated by some other process, such as lysosomal digestion, and that there may have been a selective resynthesis of the particulate enzyme. This seems unlikely, but may be evaluated by the administration of various agents which interfere with protein synthesis.

The reserpine-induced depletion of adrenal catecholamines has two dose-related components (13, 19–30): (a) a direct effect, at both high and low doses, on the catecholamine uptake mechanism of the storage vesicles (31, 32), and (b) a neurogenic component involved when higher doses of reserpine are used. With reserpine doses of 1 mg/kg there was almost complete depletion of the adrenal catecholamines and a decrease in dopamine β-hydroxylase activity, which was very similar to the losses observed after neurogenic stimulation induced by insulin. After low doses of reserpine (0.25 mg/kg), or if the neurogenic component was blocked by an agent such as chlorisondamine, which inhibits ganglionic transmission, there were no losses of dopamine β-hydroxylase activity even though the loss of adrenal catecholamines was comparable to that observed after insulin treatment.

The sensitivity of different animals to reserpine, even within the same species, appears to be quite variable and dependent upon the conditions under which the animals are housed and the manner in which they are handled. We had previously found (13), using the same strain of New Zealand white rabbits, that 1 mg/kg doses of reserpine produced little or no neurogenic response, as evidenced by the appearance of the animals and by the fact that there was no decrease in the dopamine β-hydroxylase content of the adrenal glands. In the same animals, reserpine doses of 5 mg/kg caused decreases in dopamine β-hydroxylase similar to those observed after insulin treatment. The rabbits used in the experiments reported here were housed for at least 1 week before treatment in new quarters which were much quieter than those used previously. Under these conditions the animals appeared to be more sensitive to reserpine.

The work from several laboratories has elucidated the general process which occurs during secretion from the adrenal medulla. Electron microscope studies have suggested that the storage vesicles fuse with the plasma membrane and extrude their contents directly to the exterior of the cell (8, 9, 33). Douglas and co-workers demonstrated that Ca^{++} ions were required for stimulation-secretion coupling (34) and that the ATP contained within the storage vesicles was secreted simultaneously with the catecholamines (1, 2). Shortly thereafter, several laboratories reported that the proteins contained within the storage vesicles, but not cytoplasmic proteins, were also secreted with the catecholamines (3–7). Among the proteins secreted was dopamine β-hydroxylase (18). These studies provided compelling evidence that secretion occurs by a process of exocytosis, but gave little information on the fate of the "empty" vesicles. Using perfused bovine adrenal glands, Poisner *et al.* (12) and Malamed *et al.* (10) obtained evidence which suggested that the vesicle membranes remained

within the cell for at least a short period following secretion. Viveros *et al.* (13) reported that following neurogenic stimulation *"in vivo"* there was a decrease in the total dopamine β-hydroxylase activity of the gland, but also an increase in the ratio of dopamine β-hydroxylase to catecholamines in the storage vesicles, suggesting that the fraction of the enzyme firmly bound to the storage vesicle membranes remains within the cell. The data reported here confirm and extend the above observations and show that almost all of the firmly bound dopamine β-hydroxylase is retained for at least 24 hr in the particulate fraction (P_2) of cell homogenates following secretion of catecholamines.

The ultimate fate of the storage vesicles following release of their contents is not known. It is also not known (a) whether each granule that secretes loses its total contents or a portion of its contents, (b) whether the "empty" or "partially empty" vesicles can be reutilized, or (c) whether the membranes are subsequently digested and new vesicles synthesized. Recovery of normal vesicle activity and function presumably begins after the neurogenic stimulation has been stopped, but there appears to be a lag period. Twenty-one hours after the administration of sucrose, there were slight but not statistically significant increases in the adrenaline content and in the dopamine β-hydroxylase activity, most notably in the S_2 fraction. It has been demonstrated (13) that the recovery of the ability of the storage vesicles to take up ^{14}C-adrenaline and to restore their catecholamine content is preceded by or coincident with an increase in protein synthesis, as indicated by the recovery of the dopamine β-hydroxylase levels and by an increase in the levels of tyrosine hydroxylase. If the vesicles were reused after secretion of either part or all of their total contents, recovery of normal function would require some mechanism to enable them to restore the several proteins lost during secretion. The vesicles may have the ability to synthesize their own soluble proteins, or proteins synthesized elsewhere may be able to be transported into the vesicles. Neither of these postulates seems likely. If the vesicles themselves were not reused, recovery would require the synthesis of new vesicles and their attendant soluble proteins.

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