Mechanism of Secretion from the Adrenal Medulla

IV. The Fate of the Storage Vesicles following Insulin and Reserpine Administration

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SUMMARY

Rabbits were treated with insulin and with reserpine to obtain information on the mechanism of secretion of catecholamines from the adrenal gland and on the fate of the catecholamine storage vesicles following secretion. Three and twenty-four hours after the intravenous injection of insulin (40 units/kg) there were statistically significant decreases in the contents of catecholamine and dopamine β-hydroxylase activity, and in the ability of the storage vesicles to take up ^{14}C-adrenaline. Forty-eight hours after insulin administration there was a significant recovery of the catecholamine content and the ability to take up ^{14}C-adrenaline. At this time the levels of dopamine β-hydroxylase had returned to normal limits. Four days after insulin treatment the dopamine β-hydroxylase levels were significantly greater than normal and the catecholamine content and uptake of ^{14}C-adrenaline were normal. Six days after insulin administration the dopamine β-hydroxylase levels and catecholamine content were significantly greater than the controls and the uptake was normal. Tyrosine hydroxylase levels remained at normal levels for 24 hr after insulin administration. Forty-eight hours after insulin injection the tyrosine hydroxylase levels were significantly greater than normal; at 96 hr they had increased to twice the normal levels and remained there until the end of the experimental period. Phenylethanolamine *N*-methyltransferase levels and the protein content were not affected by the insulin treatment.

Within 15 min after an injection of reserpine (1 mg/kg) the uptake was almost completely blocked, but the catecholamine content did not differ from the controls at that time or at 3 hr after the injection. Maximal depletion of catecholamines was observed 24 hr after reserpine. Thereafter the catecholamine content and ^{14}C-adrenaline uptake increased, and both were within normal limits at 8 days. The content of dopamine β-hydroxylase remained normal for 3 days after reserpine and at 8 days increased to approximately twice the normal levels.

Higher doses of reserpine (5 mg/kg) produced an effect which appeared to be a combination of neurogenic stimulation as induced by insulin and the depletion produced by lower doses of reserpine.

These data suggest that during neurogenic stimulation the entire soluble content of the storage vesicles, including dopamine β-hydroxylase, is secreted by exocytosis, leaving the vesicle membranes within the medullary cells. The depletion of catecholamines produced by low doses of reserpine (1 mg/kg) is due mainly to inhibition of the uptake mechanism rather than to neurogenic secretion.

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INTRODUCTION

Evidence accumulated during the past several years indicates that the soluble contents of the catecholamine storage vesicles (catecholamines, ATP, and protein) are released simultaneously and directly to the exterior of the cell during secretion from the adrenal medulla (1–7). Electron microscopic observations of the adrenal medulla (8–10) and chemical evidence (11, 12) suggest that the membranes of the storage vesicle, at least for a short time after secretion, remain within the adrenal medullary cells. However, these studies provide no information on the subsequent fate or physiological status of the "empty" vesicles.

Two properties of the storage vesicles suggest a means of obtaining additional information on their fate following secretion of catecholamines. Isolated intact storage vesicles obtained from the adrenal medulla (13, 14) or splenic nerves (15, 16) can accumulate catecholamines from the suspension medium by a process that is stimulated by ATP plus Mg^{2+} and inhibited by reserpine, prenylamine (Segontin), and a variety of other compounds. Thus, by comparing the abilities of the vesicles isolated from control and from stimulated glands to take up ^{14}C-adrenaline, one may obtain information on their functional status after secretion.

A second property which promises useful information is the association of the enzyme dopamine β-hydroxylase with the storage vesicles (17–19). During acetylcholine-evoked secretion from isolated perfused bovine adrenal glands, there is a parallel increase of catecholamines and of dopamine β-hydroxylase in the perfusates (20). Thus, during neurogenic secretion in intact animals one would expect a decrease in the dopamine β-hydroxylase content of the adrenal gland. In contrast, when the gland is depleted by reserpine, which by itself does not cause a release of catecholamines or dopamine β-hydroxylase in isolated, perfused bovine adrenal glands,[3] one would not expect any loss of this enzyme. By comparing the dopamine β-hydroxylase content of adrenal glands depleted of their catecholamines by neurogenic stimulation and by reserpine, one might obtain further insight into the secretory process.

It was also of interest to determine whether there were other specific changes in the medullary cells. Therefore, the levels of two other enzymes involved in the synthesis of adrenaline, tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase, were measured. These two enzymes are readily obtained in the supernatant fraction of homogenates of the adrenal glands after centrifugation at $26{,}000 \times g$ for 20 min.

Three groups of rabbits were used in the experiments described: (a) a control group, (b) a group whose adrenal glands were stimulated to secrete by insulin-induced hypoglycemia, and (c) a group treated with reserpine to deplete the adrenal glands of their catecholamines. The levels of dopamine β-hydroxylase, tyrosine hydroxylase, phenylethanolamine *N*-methyltransferase, catecholamines, and protein, as well as the ability of the homogenates to take up ^{14}C-adrenaline, were measured.

METHODS

All animals were from a strain of New Zealand white rabbits supplied by a local grower. During the experimental period, in which the animals were treated with 5 mg/kg of reserpine, the source of rabbits was changed. These rabbits were supposedly New Zealand whites, but many of them had brown markings on their ears and paws. The dopamine β-hydroxylase levels in this control group were significantly higher than the controls for the insulin-treated animals receiving 1 mg/kg of reserpine. All rabbits were young males weighing between 1.6 and 2.6 kg.

Animals receiving insulin were fasted for 24 hr and then were given 40 units of insulin per kilogram of body weight via the ear vein. The rabbits treated in this way generally went into shock within 2 hr and were allowed to remain in shock until 3 hr after the injection of the insulin. Preliminary experiments indicated that this proce-

[3] O. H. Viveros and L. Arqueros, unpublished observations.

dure gave consistent depletions of the adrenal catecholamines. With shorter time periods (2 hr) some rabbits had little depletion, and with larger time periods after insulin (4 hr) the mortality rate was markedly increased. Three hours after the injection of insulin, the animals either were killed and their adrenal glands removed, or were brought out of insulin shock by administration of 5 ml of 50% sucrose solution by stomach tube. The latter group was closely watched, and additional sucrose was given if the animals showed indications of again becoming hypoglycemic. At various time intervals after the administration of sucrose, these animals were killed and their adrenal glands were removed. For each series of assays, two or three treated animals and one control rabbit were used; the pair of glands from each animal were combined for the assays.

Rabbits which received reserpine were not fasted. The animals were killed at various time intervals from 15 min to 12 days after the intravenous injection of reserpine (1 mg/kg or 5 mg/kg), and their adrenal glands were removed. A control animal was simultaneously assayed with each two or three treated animals.

Preparation of homogenates. The rabbits were killed by a blow on the base of the skull, and their adrenal glands were immediately removed 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 in conical, all glass Potter-Elvehjem homogenizers. Aliquots were removed for catecholamine and protein determinations and for measuring the uptake of ^{14}C-adrenaline. The remainder was centrifuged at 26,000 $\times$ g for 20 min. The supernatant fraction was decanted, and the residue was resuspended in a volume of ice-cold water to give a mixture equivalent to 50 mg of fresh tissue per milliliter. Aliquots of the 26,000 $\times$ g supernatant fractions were assayed for dopamine β-hydroxylase, tyrosine hydroxylase, phenylethanolamine N-methyltransferase, catecholamines, and protein. Aliquots of the resuspended particulate fraction were assayed for dopamine β-hydroxylase, catecholamines, and protein. Aliquots of the particulate fractions from several different preparations were assayed for phenylethanolamine N-methyltransferase and tyrosine hydroxylase, but the amounts present either could not be detected or were less than 5% of the activities present in the 26,000 $\times$ g supernatant fraction.

Uptake of ^{14}C-adrenaline. The uptake of ^{14}C-adrenaline by homogenates of the adrenal gland was measured by a modification of the procedure previously described (21). The uptake of adrenaline is dependent on its concentration in the medium. Therefore, to compare the activities in homogenates of glands from different animals, the uptake was routinely assayed at three concentrations of adrenaline: at the endogenous levels, and with 2 and 4 μg of added adrenaline. Aliquots (0.2 ml) of the homogenates were incubated with 100 mM Tris-HCl buffer (pH 7.4), 1.25 mM $MgSO_4$, 0.1 mM iproniazid, 5 mM ATP (pH 7.4), 300 mM sucrose, 150,000–200,000 cpm of ^{14}C-adrenaline, and 2 or 4 μg of adrenaline added to separate mixtures in a final volume of 1.0 ml. The mixtures were incubated in air at 37° for 15 min, and the reaction was terminated by plunging the reaction vessels (centrifuge tubes) in ice and rapidly pipetting 3 ml of ice-cold, 0.3 M sucrose into each. A fourth reaction mixture, containing all the components except exogenous adrenaline, was kept in ice throughout the incubation period and served as a control. The mixtures were centrifuged for 20 min at 26,000 $\times$ g at 4°, and the supernatant fractions were decanted and assayed for total catecholamines, expressed as adrenaline (less than 5% of the total catecholamines was present as noradrenaline). The pellets were washed by resuspending them in 3 ml of ice-cold 0.3 M sucrose and allowing them to stand in ice for 30 min. The mixtures were centrifuged at 26,000 $\times$ g for 20 min and the supernatant fractions were decanted. The pellets were lysed by suspending them in 2 ml of ice-cold distilled water and vigorously stirring on a Vortex mixer. The mixtures were then centrifuged at 26,000 $\times$ g for 15 min, and the super-

natant fluids were decanted and assayed for radioactivity and adrenaline. The uptake of adrenaline was calculated from the following formula.

$$\text{Uptake } (\mu g) = \frac{\text{cpm } {}^{14}\text{C-adrenaline in lysate at } 37^\circ}{\text{avg. sp. act. of } {}^{14}\text{C-adrenaline in medium at } 37^\circ} - \frac{\text{cpm } {}^{14}\text{C-adrenaline in lysate at } 0^\circ}{\text{avg. sp. act. of } {}^{14}\text{C-adrenaline in medium at } 0^\circ}$$

The amount of radioactivity present in the wash was less than 1% of the total added radioactivity and need not be considered for this calculation. The uptake for each preparation was plotted as a function of the adrenaline concentration in the medium (Fig. 1), and the amount taken up when adrenaline was present at a concentration of 3 μg/ml was read from the graph. The results are expressed as nanograms of adrenaline taken up per 150 mg of tissue per 15 min when adrenaline was present in the external medium at a concentration of 3 μg/ml.

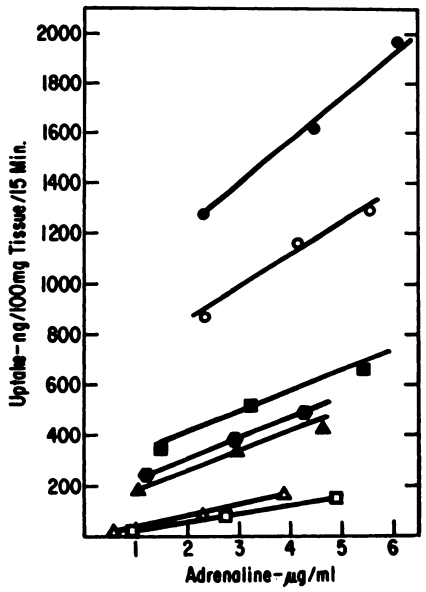

FIG. 1. *Uptake of ^{14}C-adrenaline by storage vesicles of adrenal glands*

Incubations were carried out as described in METHODS. ● and ○, Control rabbits; ■, ▲, and ⬣, 3 hr after insulin injection; □ and △, 12 hr after reserpine (1 mg/kg). The solid symbols refer to insulin-treated animals and their untreated controls; the open symbols refer to reserpine treated animals and their controls.

Assay of dopamine β-hydroxylase. Dopamine β-hydroxylase was assayed as previously described (19). The reaction mixture contained potassium phosphate buffer, pH 6.0, 100 mM; fumarate, pH 6.0, 10 mM; ascorbate, pH 6.0, 1.0 mM; ATP, pH 6.0, 5 mM; tranylcypromine, 0.5 mM; ^{3}H-tyramine (generally labeled, 100 μC/μmole), 0.01 mM; and 0.2 ml of the 26,000 × *g* supernatant fraction or 0.2 ml of the resuspended particles in a final volume of 1.0 ml. Each of the fractions was routinely assayed with three different concentrations of Cu^{2+} present. The reaction mixtures for the supernatant fraction contained 0.01, 0.025, or 0.05 mM Cu^{2+}; those for the particulate fractions contained 0.0075, 0.01, or 0.025 mM Cu^{2+}. The reaction mixtures were incubated for 15 min at 37° in an atmosphere of 100% O_2. The reaction was stopped by the addition of 1.0 ml of 7% perchloric acid. After removal of the precipitate, a 1-ml aliquot was assayed for the amount of octopamine formed by the periodate oxidation method of Friedman and Kaufman (22).

Tyrosine hydroxylase. Tyrosine hydroxylase activity was assayed as described by Nagatsu, Levitt, and Udenfriend (23). The reaction mixture contained sodium acetate buffer, pH 6.0; ferrous ammonium sulfate, 0.5 mM; tranylcypromine, 0.1 mM; 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride, 2 mM; mercaptoethanol, 20 mM; 3,5-^{3}H-tyrosine, 0.02 mM (specific activity, 50 μC/μmole); and 0.2 ml of the fraction to be assayed. The reaction mixtures were incubated in air at 37° for 15 min. The assay conditions were such that it was not necessary to correct for the very low levels of endogenous tyrosine.

Phenylethanolamine N-methyltransferase. This enzyme was assayed in the 26,000 × *g* supernatant fraction as described by

Axelrod (24), using normetanephrine as substrate. To reduce interference by endogenous catecholamines, the supernatant solution was dialyzed for 24 hr at 4° against 0.01 M acetate, pH 6.5. Under the assay conditions the amounts of metanephrine formed were linear with enzyme concentration.

Proteins and catecholamines. Aliquots of the adrenal fractions (0.1 ml) were diluted to 1.0 ml with water, and the protein was precipitated by the addition of 1.0 ml of 7% perchloric acid. The mixture was centrifuged, and the supernatant fluid was decanted, diluted, and assayed for catecholamine content without further treatment, as described previously (19). The pellet was dissolved in 1.0 ml of 0.1 N NaOH, and an aliquot was assayed for protein by the method of Lowry *et al.* (25), using bovine serum albumin as the protein standard.

Statistical methods. Since the average gland weights in several groups were different from the controls, it was necessary to express the data on a constant weight basis, applying a weight correction factor for each of the variables. The data are expressed on the basis of 150 mg of adrenal tissue, which is close to the average weight of a pair of adrenal glands. Correction factors calculated by the method of least squares were obtained from the slopes of the regression lines of the total amounts of each of the variables per pair of glands as a function of gland weights. The Student *t*-test was used to determine statistical significance. All probability (p) values are relative to the controls.

Materials. ^{14}C-Adrenaline, ^{3}H-tyramine, ^{3}H-tyrosine, and ^{14}C-methyl-*S*-adenosylmethionine were obtained from New England Nuclear Corporation. Insulin was obtained from Squibb, and reserpine was a product of Ciba Pharmaceutical Company.

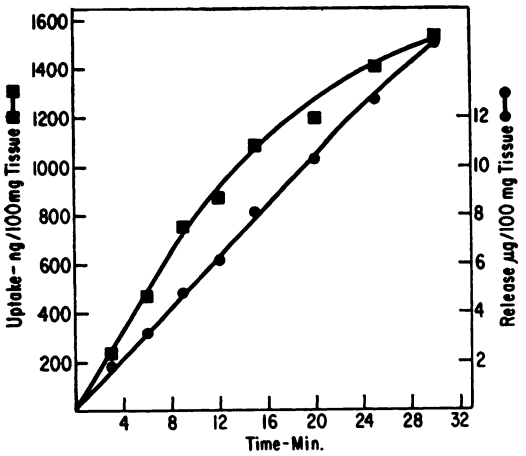

FIG. 2. *Uptake and release of adrenaline from storage vesicles of adrenal glands*

For each point the uptake was measured in duplicate at the concentration which resulted from the release of endogenous adrenaline. At 0° the amounts of adrenaline in the incubation mixtures after 3, 15, and 30 min were 2.6, 2.9, and 3.2 µg/ml, respectively. Each incubation contained the equivalent of 10 mg of tissue in a final volume of 1 ml.

RESULTS

Uptake of ^{14}C-adrenaline. Studies were carried out to establish the optimal conditions for the uptake of ^{14}C-adrenaline by homogenates of the adrenal glands. Figure 2 shows the uptake of ^{14}C-adrenaline and the release of endogenous adrenaline into the medium as a function of time. The uptake was very nearly linear for 15 min, while release was linear throughout the time of the experiment. Similar results were obtained with homogenates of glands prepared from rabbits treated with insulin.

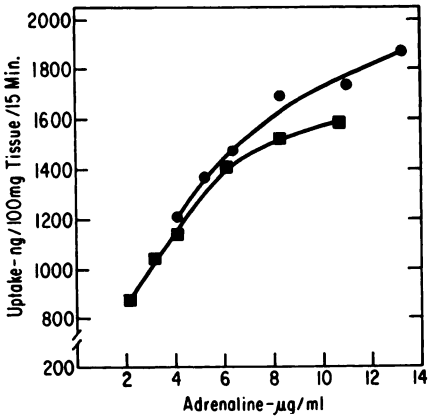

FIG. 3. *Effect of adrenaline concentration in the medium on uptake*

Each point is the average of duplicate assays. ●, Control rabbits; ■, rabbits given insulin 48 hr prior to assay.

The effect of substrate concentration on uptake is shown in Fig. 3. The absolute amounts of adrenaline taken up appear to follow typical enzyme-substrate concentration curves within the range of concentrations used. The uptake was approximately linear up to a concentration of 6 μg of adrenaline per milliliter of incubation mixture. The lowest concentrations shown are the endogenous levels. As Fig. 3 shows, the uptake of adrenaline by the homogenates of glands from insulin-treated animals (48 hr) was not significantly different from that of control animals.

The concentrations of Mg^{2+} and ATP required for optimal uptake were also investigated, and those listed under METHODS were found most suitable. Iproniazid had no effect on the uptake but was used to minimize losses of adrenaline through oxidation by monoamine oxidase. Tranylcypromine was tested as a possible substitute for iproniazid, but it markedly inhibited the uptake of adrenaline.

Effects of treatment with insulin. The effects of insulin treatment on catecholamine content, on dopamine β-hydroxylase activity, and on uptake of ^{14}C-adrenaline by the adrenal gland are shown in Table 1 and Fig. 4. Three hours after the administration of insulin there was a decrease in the catecholamine content, dopamine β-hydroxylase activity, and uptake of ^{14}C-adrenaline. These remained at approximately the same levels up to 24 hr. Forty-eight hours after insulin the catecholamine content was still lower than normal, but the dopamine β-hydroxylase activity and the uptake of ^{14}C-adrenaline did not differ from their control values. At 96 hr the dopamine β-hydroxylase activity was greater than its control level, but both the catecholamine content and the ^{14}C-adrenaline uptake were normal. At 144 hr after insulin, both the dopamine β-hydroxylase activity and the catecholamine content were greater than their control levels. There were no statistically significant differences in the protein content or in the gland weights between the control and insulin-treated groups (Table 1).

The percentages of total dopamine β-hy-

TABLE 1

Effect of insulin injection on catecholamine content, dopamine β-hydroxylase activity, and uptake of ^{14}C-adrenaline

The data are expressed on the basis of 150 mg of adrenal gland as described in METHODS. The units are as follows: catecholamines, micrograms per 150 mg; dopamine β-hydroxylase, nanomoles of octopamine formed per 150 mg per hour; ^{14}C-adrenaline uptake, nanograms per 150 mg of tissue in 15 min; protein, milligrams per 150 mg of tissue. Values are the means ± standard error for the number of animals indicated.

Time	No. of animals	Catecholamines	Dopamine β-hydroxylase	^{14}C uptake	Protein	Particulate content: Catecholamines % of Total	Particulate content: Dopamine β-hydroxylase % of Total
hr							
0	14	83 ± 4.6	18.5 ± 0.9	1186 ± 78	18.7 ± 0.5	83 ± 1.4	50 ± 1.5
3	13	36 ± 1.8[a]	14.2 ± 1.2[b]	648 ± 39[a]	17.1 ± 0.6	82 ± 1.3	40 ± 2.1[c]
24	6	37 ± 3.2[a]	15.2 ± 0.9[d]	767 ± 46[b]	20.7 ± 0.9	82 ± 2.8	53 ± 2.5
48	5	50 ± 7.5[e]	22.0 ± 2.7	886 ± 139	19.5 ± 0.7	82 ± 1.0	50 ± 2.8
96	6	96 ± 2.5	23.7 ± 1.3[b]	1211 ± 49	17.5 ± 1.2	82 ± 0.9	52 ± 1.9
144	4	126 ± 8.1[a]	23.7 ± 2.1[c]	1464 ± 55	20.3 ± 1.1	84 ± 0.7	50 ± 5.3

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

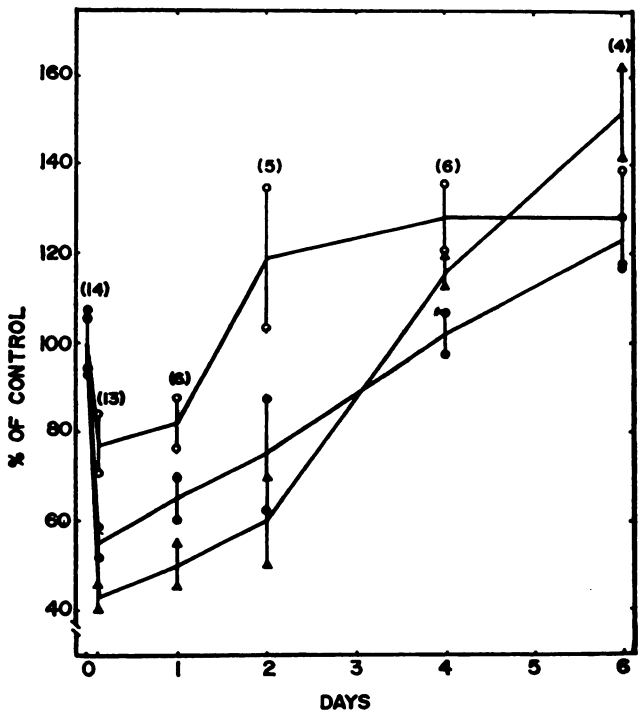

FIG. 4. *Effect of insulin treatment on catecholamine content, dopamine β-hydroxylase activity, and uptake of ^{14}C adrenaline by adrenal glands*

The data are from Table 1. The number of animals in each group is given in parentheses. The values are the means ± standard error. △, Catecholamine content; ○, dopamine β-hydroxylase activity; ●, uptake of ^{14}C-adrenaline.

droxylase activity and total adrenaline content present in the particulate fraction are also shown in Table 1. With the exception of the 3-hr time interval, the dopamine β-hydroxylase activity in the control and insulin-treated groups was equally distributed between the 26,000 × *g* supernatant fraction and the 26,000 × *g* pellet. At 3 hr the percentage of the total dopamine β-hydroxylase found in the particulate fraction was lower than that found at any other time. In all groups approximately 82% of the total adrenaline present was found in the 26,000 × *g* pellet, indicating a uniform degree of homogenization and stability of the storage vesicles in all groups.

The effect of insulin injection on the dopamine β-hydroxylase activity and the catecholamine content of the particulate fractions of adrenal homogenates is shown in Table 2. Three hours after insulin injection there was a decrease in the dopamine β-hydroxylase activity; at 96 hr this activity was greater than the control levels. The catecholamine content was lower at 3, 24, and 48 hr and higher at 144 hr than the control levels. Of special significance is the ratio of dopamine β-hydroxylase to catecholamines (see DISCUSSION); at 3, 24, and 48 hr the ratio was greater than the control levels.

The effects of insulin treatment on tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase activities are shown in Fig. 5. The phenylethanolamine *N*-methyltransferase activities of the insulin-treated animals did not differ significantly from the controls. The tyrosine hydroxylase levels remained within normal limits for 24 hr and then rose to values above normal at 48 hr; they increased further to approximately twice normal at 96 hr and remained at that level until the end of the experimental period. DeQuattro *et al.* (26) recently re-

TABLE 2

Effect of insulin on dopamine β-hydroxylase activity and catecholamine content of particulate fraction of adrenal homogenates

The values were calculated from the data of Table 1 and represent the catecholamine content (micrograms) and dopamine β-hydroxylase activity (nanomoles of octopamine formed per hour) in the particulate fraction derived from 150 mg of adrenal gland.

Time	No. of animals	Dopamine β-hydroxylase (A)	Catecholamines (B)	A:B
hr				
0	14	9.2 ± 0.5	69 ± 4	0.14 ± 0.01
3	13	5.7 ± 0.7[a]	29 ± 1.5[a]	0.20 ± 0.02[b]
24	6	8.2 ± 0.9	30 ± 3[a]	0.29 ± 0.04[a]
48	5	11.1 ± 1.5	42 ± 7[b]	0.27 ± 0.03[a]
96	6	12.2 ± 0.5[a]	78 ± 2	0.16 ± 0.01
144	4	12.0 ± 1.9	106 ± 6[a]	0.12 ± 0.02

[a] $p < 0.001$.
[b] $p < 0.01$.
[c] $p < 0.005$.

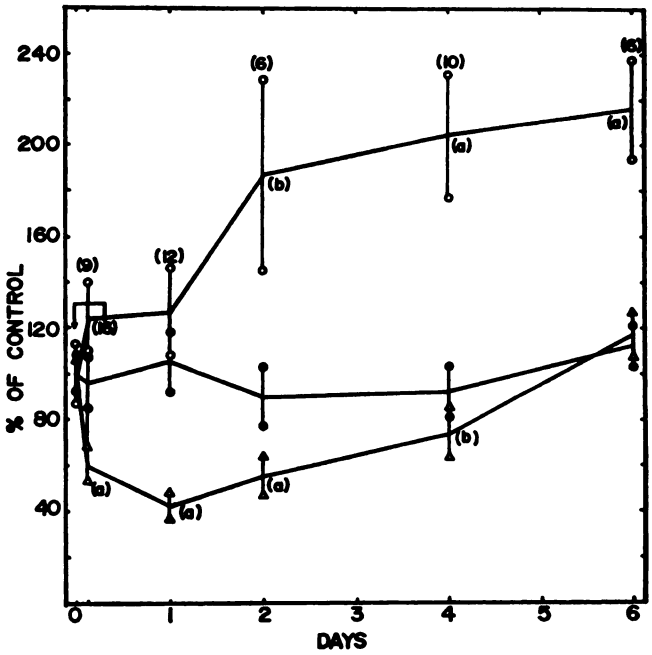

FIG. 5. *Effect of insulin treatment on catecholamine content, tyrosine hydroxylase activity, and phenylethanolamine N-methyltransferase activity of adrenal glands*

The number of animals in each group is shown in parentheses. The letters in parentheses refer to the following significant p values (Student's t-test): (a) $p < 0.001$; (b) $p < 0.02$. The means ± standard errors per 150 mg of tissue for the controls are: catecholamines (△), 92 ± 4 μg; tyrosine hydroxylase (○), 23.8 ± 2.8 mμmoles of dopa formed per hour; phenylethanolamine N-methytransferase (●), 27.9 ± 1.9 mμmoles of metanephrine formed per hour.

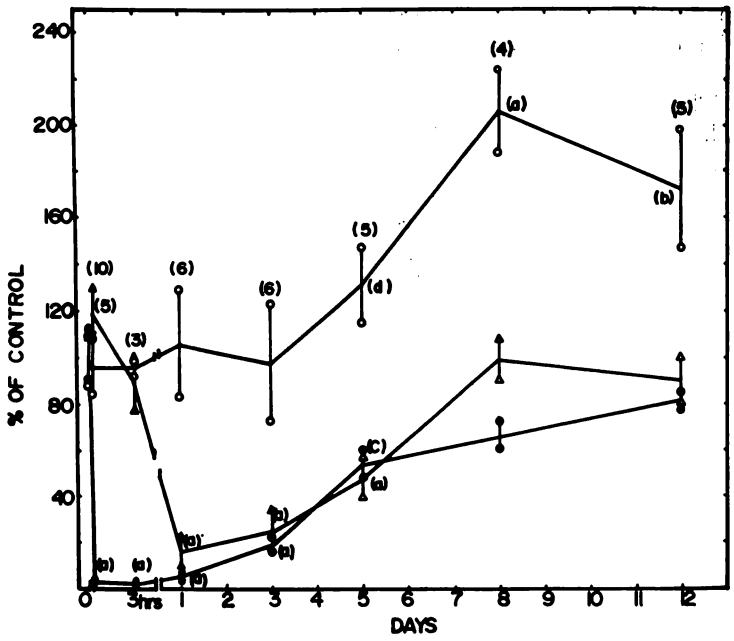

FIG. 6. *Effect of reserpine (1 mg/kg) on catecholamine content, dopamine β-hydroxylase activity, and uptake of ^{14}C-adrenaline by adrenal glands*

The number of animals in each group is shown in parentheses. The letters in parentheses refer to the following significant p values (Student's t-test): (a) $p < 0.001$; (b) $p < 0.005$; (c) $p < 0.02$; (d) $p < 0.05$. The means ± standard errors per 150 mg of tissue for the control values are: catecholamines (△), 89 ± 7 μg; dopamine β-hydroxylase (○), 23.3 ± 1.8 mμmoles of octopamine formed per hour; ^{14}C-adrenaline uptake (●), 1752 ± 190 mμg/15 min.

ported similar increases in the tyrosine hydroxylase activity of the heart and adrenal glands 24 hr after sino-aortic denervation. It should be noted that during the 24–48-hr period after insulin, the adrenal gland showed marked recovery of its catecholamine content, its ability to take up ^{14}C-adrenaline, and its dopamine β-hydroxylase activity, and also exhibited increases in its tyrosine hydroxylase activity.

Effect of reserpine. The ^{14}C-adrenaline uptake, dopamine β-hydroxylase activity, and catecholamine content of the adrenal glands in response to the administration of 1 mg/kg of reserpine are shown in Fig. 6. Within 15 min after the injection of reserpine the uptake of ^{14}C-adrenaline was almost completely inhibited, but there was no significant change in the dopamine β-hydroxylase activity or the catecholamine content of the gland. Twenty-four hours after reserpine, the catecholamine content reached a minimal level and remained at approximately the same level up to 72 hr. Thereafter both the ability to take up ^{14}C-adrenaline and the catecholamine content rose, and by the 8th day the values were not significantly different from the controls. The dopamine β-hydroxylase activity remained at normal levels for 3 days after reserpine and then increased. Five days after reserpine, the levels of this enzyme were significantly greater than the control values; at 8 days they were approximately twice the normal values and subsequently declined. In the reserpine-treated rabbits the distribution of dopamine β-hydroxylase and catecholamines between the particulate fraction and the supernatant fraction did not differ from that of the controls, with one exception. The particulate fraction of the controls contained 55 ± 3% of the total dopamine β-hydroxylase activity and 82 ± 2% of the total catecholamine content.

At 5 days the particulate fraction contained 68 ± 1% of the dopamine β-hydroxylase activity and 83 ± 1% of the total catecholamine content. There were no differences in the weights of the adrenal glands or in their protein content.

The response to a dose of 5 mg/kg of reserpine is shown in Fig. 7. This higher dose level was used because of the apparent contradiction of the results obtained at a dose of 1 mg/kg of reserpine with those reported by Lundborg (27), who found that rabbits treated with 5 mg/kg of reserpine recovered their normal ability to take up ^{14}C-adrenaline within 48 hr even though their adrenal catecholamine content remained almost depleted. In our experiments the recovery of catecholamine content and the recovery of the ability to take up ^{14}C-adrenaline from the medium closely paralleled each other and did not reach normal levels until 8 days after the administration of reserpine.

At the higher dose of reserpine, there was a decrease in the dopamine β-hydroxylase levels at 3 hr. Thereafter the levels of this enzyme fell only slightly, and then increased to normal values at 5 days. In contrast, the catecholamine content fell from 40% of the control value at 3 hr to 2% of the control value at 24 hr. Five days after reserpine, the catecholamine content and the uptake of ^{14}C-adrenaline were approximately 30% of the control values. The decrease in the dopamine β-hydroxylase levels after administration of 5 mg/kg of reserpine was similar to the decrease observed after insulin-induced secretion and was probably due to increased splanchnic stimulation as a result of the high dose of reserpine.

DISCUSSION

The response of the adrenal medulla to insulin is distinctly different from its response to reserpine (Figs. 4 and 6). These

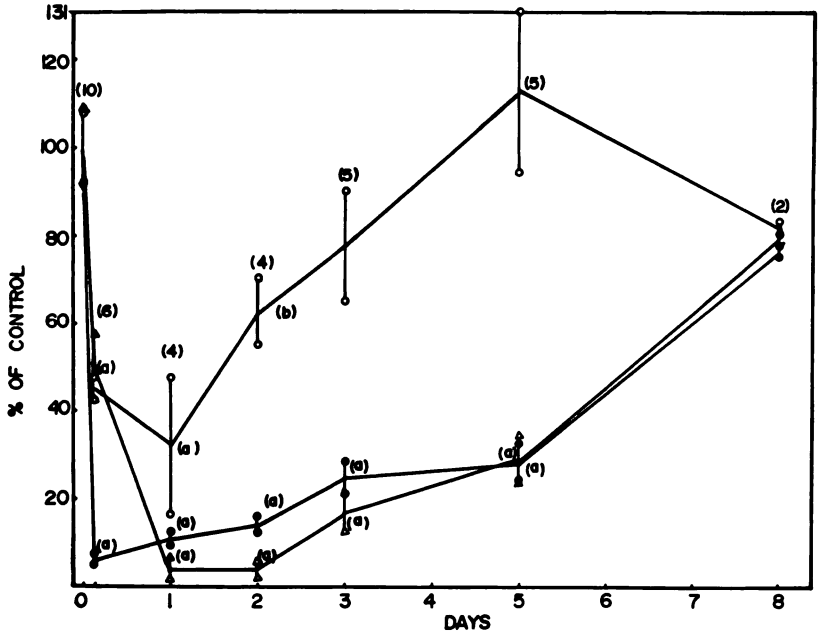

FIG. 7. *Effect of reserpine (5 mg/kg) on catecholamine content, dopamine β-hydroxylase activity, and uptake of ^{14}C-adrenaline in adrenal glands*

The number of animals in each group is shown in parentheses. The letters in parentheses refer to the following p values (Student's t-test): (a) $p < 0.001$; (b) $p < 0.01$. The means ± standard errors per 150 mg of tissue for the control values are: catecholamines (△), 102 ± 8 μg; dopamine β-hydroxylase (○), 40.7 + 2.8 mμmoles of octopamine formed per hour; ^{14}C-adrenaline uptake (●), 1502 ± 126 mμg/15 min.

observations are consistent with the previously known actions of reserpine and with the recent report that acetylcholine stimulates the release of dopamine β-hydroxylase in isolated, perfused bovine adrenal glands (20). Reserpine blocks the uptake of catecholamines into storage vesicles (13, 14). As a consequence, the adrenal glands cannot maintain their catecholamine stores and depletion occurs. In contrast, insulin causes hypoglycemia, which induces reflex neurogenic stimulation of the adrenal gland that results in the secretion of adrenaline and noradrenaline. During the secretory process the soluble contents of the storage vesicles are released (1–7). Because dopamine β-hydroxylase is, in part, a soluble component of the storage vesicles (19, 20, 28), its content in the gland is decreased. Our data show that the dopamine β-hydroxylase content of insulin-stimulated glands is less than that of control glands, suggesting that this enzyme is released *in vivo* as well as from isolated, perfused adrenal glands (20). The unchanged levels of dopamine β-hydroxylase in reserpine-treated (1 mg/kg) animals during the depletion phase and the distribution of this enzyme between the particulate and supernatant fractions indicates that the storage vesicles remain intact and retain their soluble protein components even though they have lost the ability to maintain their catecholamine stores.

The depletion of the catecholamine content of the adrenal gland by reserpine is much more rapid than one would expect if depletion were due solely to the failure to resynthesize catecholamines lost through normal secretion. The turnover time of adrenal catecholamine in rabbits is about 8–10 days (29). Thus one would not expect to find 75–80% depletion in 24 hr. In addition, the observation that the content of dopamine β-hydroxylase remained within normal limits following the injection of 1 mg/kg of reserpine while the catecholamine level decreased to 14% of the control suggests that the depletion was not due to neurogenically stimulated release. The rapid depletion observed following reserpine treatment can be explained on the basis of the ability of the drug to inhibit the uptake of adrenaline, noradrenaline, and dopamine by the storage vesicles. In the intact cell, the catecholamines in the storage complex within the vesicles probably exist in a dynamic steady state with "free" catecholamines in the cytoplasm. Under normal conditions the amounts of "free" catecholamines in the cytoplasm would be maintained at a low level because the storage vesicles can take up the cytoplasmic catecholamines by the Mg^{2+} + ATP-dependent mechanism (13, 14). When this uptake is blocked by reserpine, the cytoplasmic catecholamines increase and either are metabolized or diffuse out of the medullary cells, leading to a rapid depletion of the storage vesicles. This depletion is further enhanced by blocking the access of dopamine to dopamine β-hydroxylase within the storage vesicles. In our experiments, 5 mg/kg of reserpine caused a depletion of both catecholamines and dopamine β-hydroxylase, suggesting that there was increased neural stimulation. This was also indicated by the appearance and behavior of the rabbits. Within 2–3 min after the injection of reserpine the animals became hyperactive, frequently went into convulsions, and subsequently died. The mortality rate was 30–40%. The rabbits that received 1 mg/kg of reserpine remained outwardly normal for several hours, and there were no deaths among this group.

Whether or not there is a neurogenic contribution to the depletion of adrenal catecholamines caused by reserpine appears to be related to the amount of the drug administered and to the species of animal employed. In cats receiving doses of 0.4–0.7 mg/kg (30) or 1–2.5 mg/kg of reserpine (31) the catecholamine content of the intact right adrenal gland was markedly lowered, but the content of the denervated left adrenal gland was within the range of values obtained in the adrenal glands of cats not given reserpine. Stjärne and Schapiro (32) found that reserpine (3 mg/kg, intravenously) increased the resting secretion level from denervated cat adrenal glands as well as from intact adrenal glands (33). Eränkö and Hopsu (34) reported that a single in-

jection of 1.25–2.5 mg/kg of reserpine in rats caused a selective depletion of noradrenaline and that this effect was abolished by denervation. Twenty-four hours after the administration of reserpine (10 mg/kg) to rats whose left adrenal glands had been denervated, the catecholamine content of the intact gland decreased 76% and the content of the denervated gland decreased 36% (35). After repeated doses of reserpine (1 mg/kg) for 3 or 5 days, Callingham and Mann (36) and Mirkin (37) found that the denervated glands of rats were depleted of their catecholamines to the same extent as the intact contralateral gland. Taketomo *et al.* (38) reported that reserpine (5 mg/kg) caused almost a complete depletion of the catecholamine content of rabbit adrenals in 16 hr. Section of the spinal cord at the first thoracic segment blocked the release of catecholamines induced by reserpine. Kroneberg and Schumann (39) and Carlsson *et al.* (40) demonstrated that denervation reduced but did not prevent the loss of catecholamines from rabbit adrenals after 2 or 5 mg/kg of reserpine. These and other studies clearly establish that an intact nerve supply is not necessary for the depleting action of reserpine on the adrenal gland and indicate that the neurogenic contribution is related to the amount of drug administered in a given species. In the presence of an intact nerve supply, the depleting effect of reserpine is enhanced because of the more rapid secretion of catecholamines.

The following four models have been proposed for the physiological secretion of catecholamines from the adrenal medulla.

1. The secretory stimulus may cause intracellular disruption of the storage vesicles and intracellular release of the storage vesicle content, followed by diffusion through the plasma membrane.

2. The catecholamine content of the vesicles is in equilibrium with a small pool of cytoplasmic catecholamines. The secretory stimulus allows some of the cytoplasmic catecholamines to diffuse through the plasma membrane. After cessation of the stimulus, equilibrium is again established between the catecholamine contents in the storage vesicles and in the cytoplasm. This process implies that the storage vesicles remain intact after secretion.

3. Secretion occurs by a process in which the entire storage vesicle is extruded into the intercellular space.

4. Secretion occurs by exocytosis, a process by which the entire soluble contents of the storage vesicles are extruded directly to the exterior of the cell, leaving the vesicle membrane either free within the cell or incorporated into the plasma membrane.

Evidence from several laboratories strongly supports exocytosis as the secretory mechanism. It has been shown the catecholamines, adenine nucleotides, and protein contained within the storage vesicles are simultaneously released in the same relative amounts as in isolated, intact storage vesicles (1–7), but that cytoplasmic protein, cholesterol, and phospholipid are not released during secretion (7, 10, 11). This information indicates that mechanisms 1 and 2 are unlikely. Electron microscopic evidence (8–10) indicates that the storage vesicle membranes remain within the medullary cells after secretion, a model that is not consistent with extrusion of the vesicles.

The studies on the insulin-treated rabbits provide new information on the mechanism of secretion and on the subsequent fate of the storage vesicles following neurogenically induced secretion. The decrease in the ability to take up ^{14}C-adrenaline is inconsistent with model 2 as a mechanism of secretion and may be considered as evidence that this model does not apply. The data do not exclude the possibility that the vesicles may be repaired and used at a later time, but the parallel recoveries of catecholamine content, dopamine β-hydroxylase activity, and ability to take up ^{14}C-adrenaline suggest that the recoveries depend on the synthesis of new storage vesicles.

The decreased uptake may be explained in several ways: (a) extrusion of the entire vesicle during secretion, (b) release of intravesicular protein and ATP, which may be necessary for catecholamine binding, during secretion by exocytosis, (c) disruption of the vesicle membrane following

secretion by exocytosis, or (d) direct inhibition of the uptake process by treatment with insulin. The evidence cited above, and the additional evidence in the following paragraph, indicate strongly that secretion occurs by exocytosis. A direct effect on the uptake process itself as a result of insulin treatment seems unlikely. Furthermore, when reserpine was administered the uptake was completely blocked prior to a decrease in the catecholamine content. The observed correlation between decrease in ^{14}C-adrenaline uptake and decrease in catecholamine content after insulin suggest that (b) or (c) is a more likely explanation.

The data in Table 2 provide additional evidence that secretion occurs by exocytosis. The dopamine β-hydroxylase activity of the particulate fraction isolated in 0.3 M sucrose is associated with the storage vesicles, both in an apparently soluble form within the vesicles and firmly attached to the vesicle membranes (19, 28). If secretion occurs by extrusion of the entire granule, then one would expect that the dopamine β-hydroxylase to catecholamine ratio of the particulate fraction from the insulin-treated groups should be the same as that of the controls. However, 3 hr after the injection of insulin, this ratio showed a significant increase. The catecholamines had decreased to 42 ± 2% of the controls, and the dopamine β-hydroxylase activity had decreased to 62 ± 8% of the controls. At both 24 and 48 hr after insulin the ratios were still significantly greater than those of the controls, but at those times part of the dopamine β-hydroxylase activity may have been due to synthesis of new enzyme; it seems unlikely that there would have been significant synthesis of new amounts at 3 hr. The increased ratio in the particulate fraction indicates that the storage vesicle membrane remained within the medullary cells, and therefore the secretion occurred by exocytosis.

During the 24–48-hr period following insulin-induced depletion, there was a marked recovery of dopamine β-hydroxylase activity, and lesser recoveries of catecholamine content and ability to take up ^{14}C-adrenaline. At the same time, there was a significant increase in tyrosine hydroxylase. These concurrent events suggest that the syntheses of tyrosine hydroxylase, dopamine β-hydroxylase, and storage vesicles are controlled by a common regulatory factor. This common factor does not appear to be the levels of the enzymes or catecholamines, but may be related to neural stimulation or to some other factor produced as a result of the stress to the animal. Following a 5 mg/kg dose of reserpine, the decrease in dopamine β-hydroxylase indicated that part of the depletion was due to neural stimulation, and the recovery of this activity was evident during the 24–48-hr period after injection. After a depleting dose of 1 mg/kg of reserpine, however, there appeared to be no neural stimulation, and increases in dopamine β-hydroxylase activity and significant recoveries of the catecholamine content and uptake of ^{14}C-adrenaline did not begin until after the 3rd day, even though the catecholamine content of the glands was much less than that after insulin depletion.

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