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Recovery of Rat Adrenal Amine Stores after Insulin Administration

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SUMMARY

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Acute adrenal medullary discharge of catecholamines was produced in response to the hypoglycemia caused by a large dose of insulin (5 IU/kg, intravenously). Rats were killed 4, 24, 48, 72, or 96 hr after drug administration. Intact adrenal storage vesicles were separated from empty vesicles by differential and density gradient centrifugation, and all fractions were analyzed for catecholamines, dopamine β-hydroxylase (a marker for vesicle membranes), and ATP. Four hours after insulin, vesicular catecholamines and ATP fell to 25% of control levels, while vesicular dopamine β-hydroxylase fell to 40%; the enzyme activity increased in the broken vesicle membrane fraction. The ability of isolated storage vesicles to incorporate [^{14}C]epinephrine fell to about 25% of controls, while incorporation of [^{3}H]metaraminol fell only to 65%. Twenty-four hours after insulin administration vesicular ATP, dopamine β-hydroxylase, and [^{14}C]epinephrine incorporation returned to 50–60% of controls, while [^{3}H]metaraminol incorporation returned to normal; vesicular catecholamine content remained at about 25%. Catecholamine recovery consistently lagged behind all other parameters; by 96 hr vesicular catecholamines, ATP, and [^{14}C]epinephrine incorporation approached control levels, while vesicular dopamine β-hydroxylase exceeded control values. Isopycnic sucrose density gradient centrifugation of vesicles from control rats, and from rats given insulin 24 hr previously, indicated that the newly synthesized storage vesicles were able to incorporate both isotopically labeled epinephrine and metaraminol, but that the new vesicles had a lower equilibrium density than the original population of vesicles. They also had a lower catecholamine content and a lower specificity for epinephrine relative to metaraminol than in the controls. The efflux of endogenous or newly incorporated epinephrine from the new vesicles was nearly identical with that of the controls. These data suggest that the sequence of recovery of adrenal amine stores after sympathetic discharge proceeds by the following process: (a) resynthesis of vesicles (reappearance of vesicular dopamine β-hydroxylase) with restoration of ability to incorporate metaraminol; (b) restoration of the storage mechanism (ATP) and epinephrine incorporation; (c) restoration of catecholamines; (d) oversynthesis of new vesicles (as determined by dopamine β-hydroxylase measurements).

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INTRODUCTION

A large body of evidence supports the view that release of amines from the adrenal medulla proceeds by exocytosis. Stimulation

of adrenal glands either *in vivo* or *in vitro* results in the release of catecholamines and adenine nucleotides in a molar ratio of 4:1 (1–5), and of chromogranins and other soluble vesicular proteins, including dopamine β-hydroxylase (6–9).

Dopamine β-hydroxylase has proven to be a key to the determination of several aspects of secretion from the adrenal medulla, because it is associated with both the soluble and particulate (membrane) fractions of the storage vesicles (10). If secretion of the soluble contents of the vesicles occurs in an all-or-none fashion, the ratio of the enzyme to catecholamines in remaining intact vesicles should be the same as in the original population; if on, the other hand, partial secretion of the vesicle contents occurs, the remaining vesicles will have higher enzyme to catecholamine ratios. By these criteria all-or-none secretion has been demonstrated in rabbit (11) and rat (1) adrenals.

Less attention has been directed to the mechanism by which the adrenal medulla recovers its amine stores following massive depletion. The gland must synthesize vesicle proteins, adenine nucleotides, and catecholamines; it is unclear whether the empty vesicle membranes which remain in the chromaffin cell after secreting their contents are reused or whether synthesis of vesicle membranes occurs "*de novo*." Numerous studies have shown that the levels of tyrosine hydroxylase, the apparent rate-limiting enzyme in norepinephrine synthesis (12, 13), increase after neurogenic stimulation of the gland, such as that produced by insulin-induced hypoglycemia (9, 14, 15). Whether the resynthesis of catecholamines is the rate-limiting step in recovery of the amine stores, however, has not yet been determined.

Viveros and co-workers (11) have shown that, in adrenals from rabbits in whom sympathetic discharge has been elicited by insulin-induced hypoglycemia, vesicular dopamine β-hydroxylase returns to normal levels prior to the recovery of catecholamines. Thus resynthesis of storage vesicles is not the rate-limiting step in the recovery of amine stores in this species, and the rapid recovery of the enzyme results in the formation of vesicles with higher than normal enzyme to catecholamine ratios and lower equilibrium densities (11).

Additional studies have attempted to determine whether the rate-limiting step in recovery is the ability of the new vesicles to take up and store amines, and whether the intravesicular accumulation of adenine nucleotides determines the storage capabilities of the newly synthesized vesicles. Viveros and co-workers (9) found that the loss of catecholamines exceeded the loss of ability to incorporate [^{14}C]epinephrine 3 hr after insulin. There was a parallel recovery of both parameters at 24 and 48 hr after insulin, but at 96 and 144 hr the recovery of amines exceeded both the normal content of the vesicles and their ability to incorporate [^{14}C]epinephrine. Keswani and co-workers (16) found that the rate of recovery of [^{14}C]epinephrine incorporation paralleled that of epinephrine repletion in guinea pig adrenal glands; the ATP level returned to normal at the same rate. This does not agree with the data of Schümann (17), who observed a decrease in the catecholamine to ATP ratio in adrenal vesicles from rats given insulin 24 hr previously. These discrepancies among the results from various species indicate the need for a methodical study of the recovery of vesicular components and storage mechanisms.

Short-term studies of insulin-induced catecholamine secretion in the rat have suggested that vesicle resynthesis may occur quite rapidly (1). Thus, while the losses of intact vesicular catecholamines, ATP, and dopamine β-hydroxylase are parallel during the first 2 hr of secretion, there is a smaller drop in vesicular dopamine β-hydroxylase compared to catecholamines in the third hour, a phenomenon not observed in rabbits (11). This may indicate the presence of newly synthesized storage vesicles which do not have a normal catecholamine content. Furthermore, while the ability to incorporate [^{14}C]epinephrine decreases uniformly with catecholamine levels over a 4-hr period following insulin administration, the ability of the vesicles to incorporate metaraminol, a non-catecholamine, decreases only during the initial 1 or 2 hr of stimulation (1), suggesting that metaraminol may be incor-

porated into the new vesicles. Indeed, earlier studies have shown that [^{3}H]metaraminol is taken up by a different mechanism from that of epinephrine, is stored less stably than epinephrine, and may be incorporated into a pool with a lower capacity for incorporated amines than the pool into which most of the [^{14}C]epinephrine is incorporated (18–21).

The present study was undertaken to determine the sequence of events during the recovery of the various components of rat adrenal storage vesicles following massive depletion. These data can also yield information both about the nature of the rate-limiting step in the recovery of amine stores and the properties of newly synthesized vesicles.

METHODS

Male albino rats of the Sprague-Dawley strain (Holtzman Rat Company, 200–250 g) were fasted for 24 hr and given insulin (5 IU/kg) via a tail vein; they were brought out of hypoglycemic shock 4 hr later by the administration of 20% glucose (3 ml, intraperitoneally). Animals were killed by decapitation 4, 24, 48, 72, or 96 hr after insulin administration. The adrenal glands from each animal were excised, cleaned of fat and connective tissue, and homogenized in an all-glass apparatus in 2.5 ml of ice-cold sucrose-Tris (300 mM sucrose, 25 mM Tris, and 0.01 mM iproniazid, adjusted to pH 7 with H_2SO_4). The suspension was centrifuged at 800 × *g* for 10 min, and the supernatant solution was decanted. The pellet was resuspended by glass-to-glass homogenization in 5 ml of distilled water and analyzed for catecholamines and dopamine β-hydroxylase (fraction A). One milliliter of the supernatant was diluted with 1 ml of water, homogenized, and assayed (fraction B). Another milliliter of the 800 × *g* supernatant was layered over 2.5 ml of 1.6 M sucrose (pH 7) containing 500 units/ml of beef catalase (Sigma) (required for the maintenance of dopamine β-hydroxylase activity) and centrifuged for 2 hr at 140,000 × *g* in the No. 40 rotor of a Beckman model L2 ultracentrifuge. This separates intact vesicles from broken vesicle membranes (1) and from most mitochondrial and lysosomal contaminants (22). The 300 mM sucrose layer (fraction C) and the 1.6 M sucrose layer (fraction D) were diluted with water to final volumes of 2 and 4 ml, respectively, homogenized, and assayed for catecholamines and dopamine β-hydroxylase. The vesicular pellet (fraction E) was resuspended in 2 ml of water and homogenized to lyse the vesicles. One milliliter was removed for the determination of catecholamines and dopamine β-hydroxylase, and the remainder was centrifuged at 26,000 × *g* to remove the vesicle membranes; the supernatant obtained from this latter centrifugation was analyzed for ATP.

Incorporation of radioactive amines. Rats were given insulin and killed as already described. The glands from each animal were homogenized in 2.2 ml of sucrose-Tris, and an aliquot was withdrawn for catecholamine analysis. After the suspension had been centrifuged at 800 × *g* for 10 min, the supernatant was decanted, and 0.4 ml was pipetted into each of four tubes containing 0.1 ml of 50 mM ATP plus Mg^{2+}, 0.1 ml of 1 mM epinephrine, and either 1 μCi of [^{14}C]epinephrine or 5 μCi of [^{3}H]metaraminol plus 0.1 ml of 1 mM metaraminol. The added epinephrine was sufficient to eliminate any differences in extravesicular amine concentrations among the samples. Sucrose-Tris was added to each tube to give a total volume of 1 ml, and one epinephrine- and one metaraminol-containing sample were brought to 30° for 30 min; the duplicate tubes were kept at 0° for 30 min. Uptake was stopped by the addition of 2 ml of ice-cold sucrose-Tris, and the samples were centrifuged for 10 min at 26,000 × *g*. The supernatants were decanted and added to an equal volume of 7% perchloric acid, centrifuged, and analyzed for catecholamines and radioactivity. The vesicular pellet was washed with sucrose-Tris, centrifuged, rewashed, centrifuged, resuspended (glass-to-glass homogenization) in 3 ml of 3.5% perchloric acid, recentrifuged, and then analyzed for catecholamines and radioactivity. Uptake in each sample was calculated as follows, where "CA" refers to catecholamines:

Gross uptake per gland

$$= \frac{\text{cpm in vesicles} \times \text{CA content per gland}}{\text{specific activity of labeling medium} \times \text{CA content of vesicles in sample}}$$

Gross uptake per 100 μg CA

$$= \frac{\text{uptake per gland} \times 100}{\mu\text{g CA per gland}}$$

The uptake at 0° was then subtracted from the uptake at 30° to give the temperature-dependent vesicular uptakes.

Efflux of newly incorporated amines. Efflux of endogenous catecholamines and of newly incorporated [^{14}C]epinephrine from vesicles from control rats or from rats given insulin 24 hr previously was measured at 30° as described previously (1). Adrenal glands from 10 rats were homogenized in 33 ml of sucrose-Tris and centrifuged at 800 × *g* for 10 min. Fifteen milliliters of vesicle suspension were added to 5 ml of sucrose-Tris containing 20 mM ATP, 20 mM Mg^{2+}, 0.4 mM epinephrine, and 3 μCi of [^{14}C]epinephrine. The mixtures were incubated for 30 min at 30° and then placed in an ice bath to stop the uptake. After centrifugation at 26,000 × *g* for 10 min to precipitate the labeled vesicles, the pellet was washed by resuspension (Teflon-to-glass homogenization) in fresh sucrose-Tris and then centrifuged; this procedure was repeated twice more, and the pellet was resuspended in 20 ml of sucrose-Tris. Sets of fifteen 1-ml aliquots of labeled vesicles were brought to 30° to allow efflux to occur, and efflux was stopped by the addition of 2 ml of ice-cold sucrose-Tris after 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. Samples were centrifuged for 10 min at 26,000 × *g*, and the supernatant solution was decanted and analyzed for catecholamines and radioactivity. The vesicular pellets were resuspended in 3 ml of 3.5% perchloric acid (to lyse the vesicles), centrifuged for 10 min at 26,000 × *g* (to remove precipitated proteins), and analyzed for catecholamines and radioactivity. The effluxes of endogenous catecholamines and [^{14}C]epinephrine were calculated as described previously (20).

Continuous sucrose density gradients. Continuous density gradients, hyperbolic from 1 to 2 M sucrose, were prepared with a modification (21) of the apparatus of Ayad *et al.* (23). Sucrose (1 M) was pumped into a mixing chamber containing 2 M sucrose (15 ml/gradient) at a rate equal to that at which it was pumped out; the total volume of each gradient was 30 ml. Adrenal storage vesicles from control rats and from rats killed 24 hr after insulin administration were labeled with [^{14}C]epinephrine (10 mCi/mmole), [^{3}H]-metaraminol (50 mCi/mmole), or [^{3}H]epinephrine (50 mCi/mmole), washed twice, and resuspended in sucrose-Tris. Mixtures of vesicles were prepared as follows: tube 1 contained [^{14}C]epinephrine (control) + [^{3}H]metaraminol (control); tube 2, [^{14}C]epinephrine (control) + [^{3}H]metaraminol (insulin); and tube 3, [^{14}C]epinephrine (control) + [^{3}H]epinephrine (insulin).

The mixtures (total volume, 1 ml/mixture) were then layered onto the gradients and centrifuged at 105,000 × *g* for 3 hr in a Spinco SW 25 rotor. This is sufficient time to permit equilibrium density to be reached. The gradient tubes were emptied dropwise from the bottom (20 drops/sample), and each sample was diluted with 2 ml of 5% perchloric acid, centrifuged at 26,000 × *g* for 10 min, and analyzed for ^{14}C, ^{3}H, and catecholamines.

Assays. For catecholamines, 0.1-ml aliquots of all samples were added to 1.9 ml of 3.5% perchloric acid and centrifuged at 26,000 × *g* 10 min in order to remove precipitated protein. The supernatants were analyzed for catecholamines by the trihydroxyindole method, as described previously (20), and reported as micrograms or nanomoles equivalent of epinephrine.

Radioactive amines were measured by liquid scintillation spectrometry. One milliliter of each sample was added to 10 ml of a 1:2 mixture of Triton X-100 detergent and toluene (containing 2,5-diphenyloxazole and *p*-bis[2-(5-phenyloxazolyl)]benzene).

ATP was analyzed by the luciferin-luciferase method (24). The contents of a vial of buffered firefly lantern extract were dissolved in 5 ml of water; 0.2 ml of enzyme, 0.5 ml of water, and 0.2 ml of sample were used in each assay. Phosphorescence was determined in a Farrand spectrofluorometer 20 sec after addition of the ATP-containing sample.

There was a linear relationship between sample dilution and phosphorescence, indicating that no endogenous inhibitors or quenchers were present.

Dopamine β-hydroxylase was assayed using [^{3}H]tyramine, as described previously (15); assays were done on the same day that the rats were killed. The incubations lasted 1 hr, over which time the reaction rate was linear. *p*-Hydroxymercuribenzoate was used to inactivate endogenous inhibitors (25); optimal concentrations of the mercurial were: fraction A, 1 mM; fractions B and C, 0.5 mM; fraction D, 0.025 mM; fraction E, none.

Statistical analyses. Data are reported in terms of control values, percentages of controls, and standard errors of the mean. Levels of significance were calculated by Student's *t*-test, and straight lines were determined by linear regression analysis (26).

Materials. [7-^{14}C]Epinephrine (40 mCi/mmole), [7-^{3}H]epinephrine (15 Ci/mmole), [7-^{3}H]metaraminol (15 Ci/mmole), and [G-^{3}H]tyramine (5 Ci/mmole) were obtained from New England Nuclear Corporation. Buffered firefly extract was obtained from Worthington Biochemical Corporation, and regular insulin (80 units/ml) from Squibb. Epinephrine bitartrate was obtained from Winthrop Laboratories, and metaraminol bitartrate from Merck Sharp & Dohme.

RESULTS

Isolation of storage vesicles. The subcellular distributions of catecholamines, dopamine β-hydroxylase, and ATP from adrenal glands of untreated rats are shown in Table 1. Approximately 15–18% of the total catecholamines and dopamine β-hydroxylase were found in the 800 × *g* pellet; this probably represents the fraction of cells not disrupted during homogenization, and was fairly consistent from preparation to preparation. Of the amount placed on the discontinuous gradient, about 16% of the catecholamines and dopamine β-hydroxylase were found associated with fraction C (0.3 M sucrose), indicating that about one-sixth of the vesicles were lysed during the preparation. Only 6% of the catecholamines and less than 2% of the dopamine β-hydroxylase were found in the 1.6 M sucrose (fraction D) layer; the intact vesicular pellet (fraction E) contained about 65% of the catecholamines and dopamine β-hydroxylase placed on the gradient, or about 55% of the contents of the whole gland. Total recoveries of catecholamines and dopamine β-hydroxylase from the discontinuous gradient averaged about 85% of the amount in fraction B. These values for subcellular distribution agree closely with those of Smith and Winkler (22) and of Viveros *et al.* (11).

ATP was assayed in the intact vesicular pellet (fraction E); control values averaged 9 nmoles/gland. Catecholamines in fraction E averaged 36 nmoles/gland. Thus the molar ratio of catecholamines to ATP was 4:1, which is the theoretical ratio in the storage complex. ATP values in other subcellular fractions were small and quite variable, probably because of hydrolysis of extravesicular ATP by ATPases.

Catecholamine, dopamine β-hydroxylase, and ATP distributions after insulin. Four hours after insulin administration catecholamine levels in all subcellular fractions were substantially decreased from controls (Table 1). Total catecholamines and the levels in fractions A and B (800 × *g* pellet and supernatant) were about 30% of controls; the percentage in the intact vesicles (fraction E) was somewhat lower than in fractions A and B, while the percentages in fractions C and D (0.3 M and 1.6 M sucrose) were somewhat higher. ATP in the intact vesicle fraction decreased parallel to catecholamines: the difference between depletion of ATP and catecholamines in fraction E was not significant ($p > 0.05$).

Four hours after insulin administration dopamine β-hydroxylase content of the gland was only slightly decreased, but the distribution was shifted markedly (Table 1). The enzyme level in fraction A increased slightly, while that in fraction B decreased to about 80% of control. The content in fraction C (empty vesicle membranes) doubled. As was seen with catecholamines, the dopamine β-hydroxylase level in fraction E decreased substantially, but there was a significant ($p < 0.01$) difference between the decrease in

TABLE 1

Subcellular distribution of catecholamines, dopamine β-hydroxylase, and ATP

Results are expressed as means ± standard errors.

Catecholamines							
Time after insulin	A[a]	B	C	D	E	Total (A + B)	No. of animals
Control (nmoles/gland)	10.0 ± 0.5	57.9 ± 2.2	9.5 ± 0.5	3.7 ± 0.2	35.7 ± 1.7	67.8 ± 2.7	19
hr				*% control*			
0	100 ± 5	100 ± 4	100 ± 5	100 ± 4	100 ± 5	100 ± 4	19
4	31 ± 3	28 ± 4	44 ± 4	44 ± 5	21 ± 3	28 ± 1	6
24	31 ± 3	37 ± 3	50 ± 3	60 ± 6	24 ± 2	36 ± 3	6
48	49 ± 5	52 ± 3	57 ± 4	85 ± 8	43 ± 4	51 ± 3	6
72	48 ± 5	55 ± 3	82 ± 6	87 ± 4	46 ± 2	54 ± 3	6
96	69 ± 2	81 ± 3	66 ± 4	99 ± 6	78 ± 4	79 ± 3	6

Dopamine β-hydroxylase							
Time after insulin	A	B	C	D	E	Total (A + B)	No. of animals
Control (nmoles/gland/hr)	0.19 ± 0.01	0.87 ± 0.04	0.15 ± 0.02	<0.02	0.56 ± 0.04	1.06 ± 0.05	19
hr				*% control*			
0	100 ± 5	100 ± 5	100 ± 13		100 ± 6	100 ± 5	19
4	107 ± 3	78 ± 7	207 ± 36		41 ± 4	83 ± 6	6
24	62 ± 5	62 ± 3	135 ± 14		54 ± 9	62 ± 3	6
48	87 ± 2	82 ± 2	110 ± 6		62 ± 3	84 ± 1	6
72	107 ± 3	95 ± 3	154 ± 9		75 ± 3	96 ± 3	6
96	132 ± 10	122 ± 4	152 ± 10		115 ± 2	124 ± 5	6

ATP			
Time after insulin		E	No. of animals
Control (nmoles/gland)		8.96 ± 0.44	19
hr		*% control*	
0		100 ± 5	19
4		29 ± 2	6
24		55 ± 4	5
48		67 ± 5	6
72		62 ± 3	6
96		88 ± 5	6

[a] Fraction A is the 800 × *g* pellet; fraction B is the 800 × *g* supernatant; fractions C–E were obtained on a discontinuous sucrose density gradient [C = 0.3 M sucrose layer (broken vesicle membranes), including the C/D interface; D = 1.6 M layer; E = vesicular pellet (intact vesicles)].

TABLE 2

Incorporation of amines by crude preparation of adrenal storage vesicles

Results are expressed as means ± standard errors.

Time after insulin	Catecholamines	Epinephrine incorporation		Metaraminol incorporation		No. of animals
		Per gland	Per 100 μg of catecholamines	Per gland	Per 100 μg of catecholamines	
Control	59.0 ± 2.7[a]	1.9 ± 0.1[b]	18 ± 1[c]	0.35 ± 0.02[b]	3.3 ± 0.3[c]	18
hr			*% control*			
0	100 ± 5	100 ± 5	100 ± 6	100 ± 6	100 ± 9	18
4	22 ± 2	22 ± 5	93 ± 14	67 ± 7	300 ± 29	6
24	27 ± 4	58 ± 5	260 ± 43	94 ± 7	381 ± 98	5
48	39 ± 6	61 ± 6	173 ± 16	106 ± 11	226 ± 37	5
72	56 ± 3	82 ± 6	152 ± 9	87 ± 4	139 ± 5	6
96	88 ± 10	89 ± 9	116 ± 14	98 ± 7	101 ± 13	6

[a] Nanomoles per gland.

[b] Nanomoles per gland per 30 min.

[c] Nanomoles per 30 min per 100 μg of catecholamines in vesicles.

catecholamines (79 %) and enzyme (59 %) in fraction E 4 hr after insulin.

Twenty-four hours after insulin administration both total and vesicular catecholamines increased only slightly from the values at 4 hr (Table 1). Vesicular ATP and dopamine β-hydroxylase, on the other hand, increased to about 55 % of control values, despite the fact that total enzyme dropped from 83 % of control at 4 hr to 62 % of control at 24 hr. The level of enzyme in the empty vesicle membrane fraction (C) decreased markedly over the same time period (Table 1).

Forty-eight and 72 hr after insulin total adrenal catecholamines increased to 50–55 % of control values: these values were somewhat lower in fraction E (intact vesicles) and somewhat higher in fractions C and D (Table 1). During the same time period vesicular ATP and dopamine β-hydroxylase increased to values greater than 60 and 70 %, respectively (Table 1). Total dopamine β-hydroxylase recovered to normal values by 72 hr. The enzyme content in fraction C first decreased between 24 and 48 hr, and then increased markedly between 48 and 72 hr (Table 1).

By 96 hr after insulin administration total and vesicular catecholamines and vesicular ATP had returned to nearly normal levels (80–90 %) (Table 1). For the first time since 4 hr after insulin there was no significant difference between recoveries of vesicular catecholamines and vesicular ATP. Similarly for the first time, the catecholamines in fraction E had recovered to a greater extent than in fraction C (Table 1).

The pattern of dopamine β-hydroxylase recovery at 96 hr was considerably different from that of catecholamines or ATP (Table 1). In all fractions the enzyme level was significantly higher ($p < 0.05$) than control values. The intact vesicle fraction (E) showed the smallest increase above controls, while the empty membrane fraction (C) showed the largest.

Incorporation of amines. These data are reported in terms of incorporation per gland and incorporation per 100 μg of catecholamines in the vesicles. The former parameter is a measure of the number of functional vesicles in the gland, while the latter describes the ability of each vesicle to incorporate amines relative to the amount of catecholamines already present.

Storage vesicles from control rats were able to incorporate about 2 nmoles of epinephrine in 30 min (Table 2), or 20 nmoles/100 μg of catecholamines. This corresponds to about 4 % of the catecholamines present in the vesicles. Metaraminol incorporation was

considerably lower than that of epinephrine, and represented less than 1% of the endoggenous catecholamines (Table 2).

Four hours after insulin total adrenal catecholamines and epinephrine incorporation had decreased to about 20% of control; there was no change in the incorporation when expressed as nanomoles per 100 μg of catecholamines, indicating that the incorporation per intact vesicle remained the same as in control animals. Twenty-four and 48 hr after insulin, when adrenal catecholamines had recovered only to about 40% of controls, epinephrine incorporation was greater than 60% (Table 2), and consequently the incorporation per 100 μg of catecholamines was 2–3 times normal (Table 2). Seventy-two hours after insulin the adrenal catecholamine content was about 55% of controls, but epinephrine incorporation per gland was greater than 80%, and thus incorporation per 100 μg of catecholamines remained elevated. All three parameters returned to nearly normal levels after 96 hr, at which time there was no difference between total adrenal catecholamines and epinephrine incorporation (Table 2).

The pattern of loss and recovery of the ability to incorporate metaraminol was considerably different from that of epinephrine (Table 2). At 4 hr, when adrenal catecholamine levels were 20% of controls, metaraminol incorporation was nearly 70% (Table 2); consequently metaraminol incorporation per 100 μg of catecholamines tripled. Within 24 hr of insulin administration metaraminol incorporation had returned to normal levels, despite the fact that there was no recovery in adrenal catecholamines at that time (Table 2).

Efflux of amines. To determine the ability of newly synthesized storage vesicles to store catecholamines, measurements were made of the effluxes of endogenous catecholamines and of newly incorporated [^{14}C]epinephrine from storage vesicles of control rats and rats given insulin 24 hr previously (Fig. 1). There was little difference in amine efflux between vesicles from control and insulin-treated rats, and the efflux curves from both groups were typically biphasic (Fig. 1).

Continuous sucrose density gradients. The discrepancy between vesicular catecholamine and dopamine β-hydroxylase levels during secretion and recovery, and the rapid return of the ability to incorporate amines, suggested that new vesicles were rapidly synthesized after insulin-induced secretion. The higher levels of catecholamines in fractions C and D also suggested that the new vesicles might have a lower density than the original population. To test this hypothesis, vesicles from control rats were labeled with [^{14}C]epinephrine or [^{3}H]metaraminol, and vesicles from rats given insulin 24 hr previously were labeled with [^{3}H]epinephrine or [^{3}H]metaraminol; ^{3}H-labeled vesicles from control and insulin-treated rats were mixed with ^{14}C-labeled vesicles from control rats and subjected to isopycnic sucrose density gradient centrifugation. The results appear in Fig. 2. In vesicles from untreated rats the endogenous catecholamines and newly incorporated [^{14}C]epinephrine and [^{3}H]metaraminol coincided, and the ratio of ^{3}H to ^{14}C was the same in the leading and trailing edges of the peak (Fig. 2A). However, when vesicles from insulin-treated rats were labeled with [^{3}H]metaraminol and centrifuged with con-

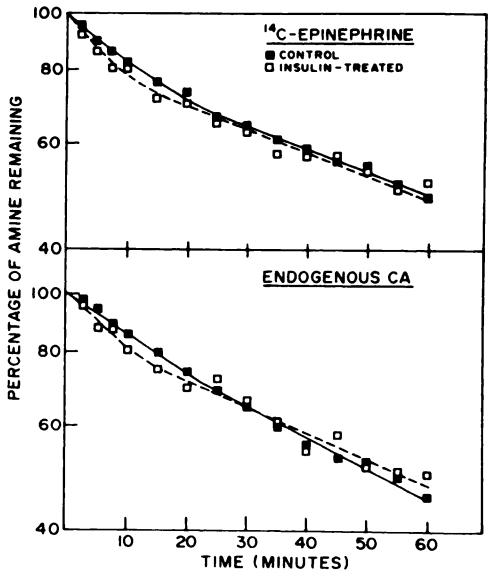

FIG. 1. *Efflux of* [^{14}C]*epinephrine and endogenous catecholamines (CA) from adrenal storage vesicles of untreated rats and of rats given insulin (5 IU/kg, intravenously) 24 hr previously*

The ordinate is logarithmic.

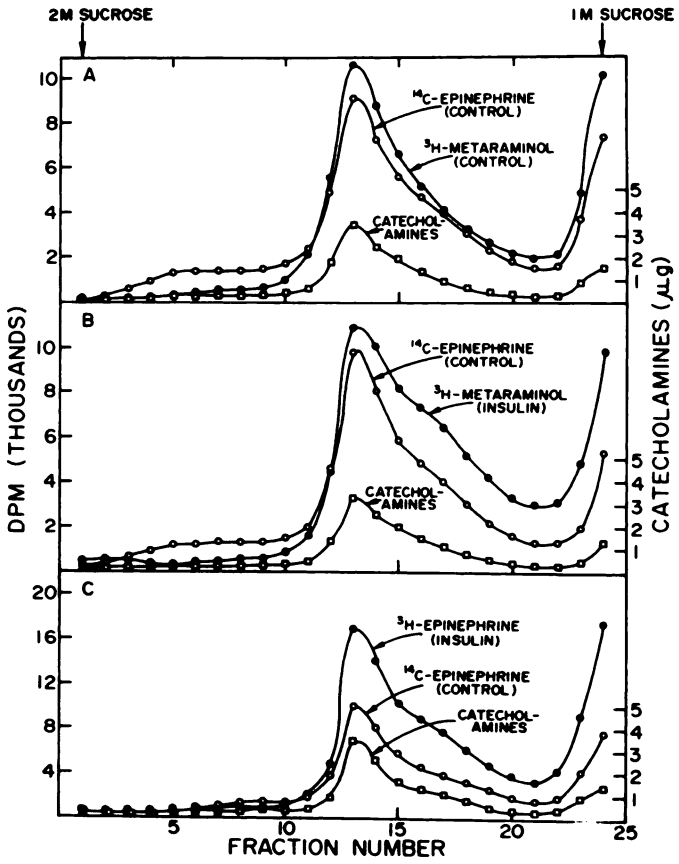

FIG. 2. *Isopycnic sucrose density gradient centrifugation of adrenal storage vesicles from untreated rats and from rats given insulin (5 IU/kg intravenously) 24 hr previously*

Vesicles were labeled with [^{14}C]epinephrine, [^{3}H]metaraminol, or [^{3}H]epinephrine and mixed as described under METHODS. A. [^{14}C]Epinephrine-labeled (○) and [^{3}H]metaraminol-labeled (●) vesicles from control rats. B. [^{14}C]Epinephrine-labeled vesicles from control rats (○) and [^{3}H]metaraminol-labeled vesicles from insulin-treated rats (●). C. [^{14}C]Epinephrine-labeled vesicles from control rats (○) and [^{3}H]epinephrine-labeled vesicles from insulin-treated rats (●). Endogenous catecholamines (□) were determined for each gradient. The leading edge in each gradient consists of fractions 11–13, and the trailing edge contains fractions 14–20. Values for ^{3}H:^{14}C ratios were computed as (trailing edge ^{3}H:^{14}C)/(leading edge ^{3}H:^{14}C) (two experiments): A, 1.01 and 0.97; B, 1.48 and 1.62; C, 1.18 and 1.16.

trol vesicles labeled with [^{14}C]epinephrine, there was an increase of 50% in the ^{3}H:^{14}C ratio in the trailing edge (Fig. 2B), indicating skewing of the [^{3}H]metaraminol distribution toward lower-density particles. This skewing is evidenced by the appearance of a "shoulder" centered at about fraction 17 [Fig. 2B]. Likewise, [^{3}H]epinephrine-labeled vesicles from insulin-treated rats centrifuged with [^{14}C]epinephrine-labeled control vesicles exhibited a shoulder near the same point, with a consequent increase of 20% in the ^{3}H:^{14}C ratio in the trailing edge compared to the leading edge (Fig. 2C).

DISCUSSION

In exocytosis the entire soluble contents of the storage vesicles are secreted to the exterior of the cell, while the vesicle membranes remain behind. Consequently, 4 hr after insulin administration there was a parallel loss of soluble components (catecholamines and ATP) and an increase in the number of empty vesicle membranes (dop-

amine β-hydroxylase in fraction C). The loss of the ability to incorporate [^{14}C]epinephrine, expressed on a per gland basis, reflected the decrease in the number of functional storage vesicles, while the unimpaired ability to incorporate epinephrine relative to the endogenous amine content indicated that the remaining vesicles were fully functional.

Although previous studies have shown that quantal release of vesicle contents occurs in both rats (1) and rabbits (11), the ratio of dopamine β-hydroxylase to catecholamines in the remaining vesicles several hours after insulin administration was found to be elevated in rats but not in rabbits. One explanation of the discrepancy between species is that the rate of new vesicle synthesis may be higher in rats; furthermore the rat probably possesses a greater relative capacity for catecholamine synthesis, since a similar species difference exists in tyrosine hydroxylase activity (9, 15). If the "extra" dopamine β-hydroxylase found at 4 hr indeed represents new vesicles, significant synthesis of vesicle protein should have occurred during that time period. Winkler and co-workers (27) have demonstrated maximal incorporation of labeled amino acids into chromogranins 4 hr after exposure of bovine adrenals to carbachol.

It remains to be determined why the new vesicles recovered their ability to incorporate metaraminol, a non-catecholamine, sooner than the ability to incorporate catecholamines. First, although metaraminol is readily incorporated by the storage vesicles, it is stored in a much less stable manner (1, 20, 21) and is incorporated by a relatively reserpine-resistant mechanism (18–21), while catecholamine uptake is reserpine-sensitive. Second, although there is a slight preference for catecholamines, protein components of the storage vesicles, such as chromogranins, are able to bind both catechol- and non-catecholamines (21), and this binding can account for about 1.5% of the total amine content of the vesicles (21). Since newly incorporated amines represent only a small percentage of the total amine content, it seems likely that metaraminol, less of which would be incorporated into the ATP storage complex, would owe much more of its observed vesicular incorporation to protein binding than would epinephrine. At a stage in recovery where new vesicles have been synthesized which do not contain ATP (4 hr) metaraminol incorporation would therefore be closer than epinephrine incorporation to control values.

If such a scheme is valid, the incorporation of [^{14}C]epinephrine should recover with a time course similar to the recovery of ATP. The measurements at 24, 48, 72, and 96 hr indicate that similarity. Furthermore, if the presence of ATP is responsible for the recovery of epinephrine incorporation, the newly incorporated epinephrine should have the same storage stability in new vesicles that it has in normal vesicles. The efflux studies from vesicles which partially recovered both their ATP and their ability to incorporate [^{14}C]epinephrine, but not their endogenous catecholamines (24 hr), indicate no difference in storage stability compared to controls. Thus the newly synthesized storage vesicles acquire the normal ability to store catecholamines as soon as their ATP content is established.

Since 21% of the dry weight of the storage vesicles is catecholamines new vesicles which lack catecholamines should have a lower equilibrium density than controls. To test this hypothesis, vesicles from control and insulin-treated rats (24 hr after insulin) were labeled with radioactive amines and subjected to isopycnic sucrose density gradient centrifugation. In control rats both [^{14}C]epinephrine- and [^{3}H]metaraminol-labeled vesicles were distributed in a fashion nearly identical with catecholamines, illustrating that both labels were incorporated into the storage vesicle fraction and that both were incorporated into vesicles of equal density. In rats given insulin, however, the distribution of [^{3}H]epinephrine-labeled vesicles was shifted to lower densities compared to controls, which is to be expected if the new vesicles lack catecholamines. [^{3}H]Metaraminol-labeled vesicles from insulin-treated rats were displaced even farther toward lower densities; vesicles which lack both catecholamines and ATP should have even lower densities than vesicles which lack only the former, and if metaraminol is incorporated into these vesicles, the distribution of metaraminol label would be shifted in that

manner. Intact vesicles which lack catecholamines and have lower densities have been observed after insulin administration in rabbits (11). The appearance of lighter storage vesicles also accounts for part of the relatively high amount of dopamine β-hydroxylase found in fraction C during recovery, although some activity was undoubtedly due to broken vesicle membranes which had not yet been degraded or reconstituted into new vesicles. Partial catecholamine repletion in the lighter vesicles could also account for the more rapid initial recovery of catecholamines in fractions C and D and the slower recovery in fraction E. These vesicles would not appear in fraction E until their densities permitted sedimentation in 1.6 M sucrose.

The pattern of dopamine β-hydroxylase levels in fraction C during recovery therefore represents the summation of two processes: destruction of broken vesicle membranes (through either proteolysis or reuse in new vesicles) and appearance of new vesicles of lighter density. The decrease in enzyme level in fraction C from 4 to 48 hr suggests that the former process continues for at least 2 days, while the later increase reflects the large amount of resynthesis of vesicles.

There is an apparent oversynthesis of storage vesicles within 96 hr of insulin administration, since dopamine β-hydroxylase levels at that time exceeded controls in all subcellular fractions. This suggests that the synthesis of new vesicles is not regulated by the number or vesicles present or by the enzyme activity itself, but instead may be determined by the catecholamine level or by the level of neural input to the gland. The importance of neural input in the rate of catecholamine recovery and tyrosine hydroxylase increase in rats given reserpine has been demonstrated by Patrick and Kirshner (29), although they did not observe as large an effect on dopamine β-hydroxylase recovery.

These data suggest that the recovery of amine stores of the adrenal medulla after insulin administration occurs in the following sequence: (a) resynthesis (or reconstitution) of vesicles with concurrent recovery of metaraminol uptake; (b) recovery of vesicular ATP with concurrent recovery of epinephrine uptake; (c) recovery of catecholamine levels. Thus the resynthesis of catecholamines is probably the rate-limiting step in recovery. While this view agrees with the earlier observation that the ratio of catecholamines to ATP in rat adrenals decreases after insulin (17), it conflicts with the data obtained in guinea pigs by Keswani and co-workers (16), who observed a more rapid recovery of norepinephrine compared to the incorporation of [^{14}C]epinephrine, and a subsequent recovery of epinephrine levels concurrently with [^{14}C]epinephrine incorporation. A possible reason for this discrepancy lies in the method of measuring incorporation; in the earlier study (16) incorporation was determined solely by incorporation of radioactivity, and the specific activity during labeling was not determined. It has been shown in several laboratories that the exogenous amine concentration during incorporation plays an important role in determining the amount incorporated, and that the exogenous amine concentration decreases if vesicles from insulin-treated animals are used (1, 9, 18–21). In our study sufficient exogenous amine (0.1 mM) was added to each incubation to obviate differences between treated and control samples; specific activity was determined for each sample to ensure that the differences in uptake were not due to differences in exogenous amine levels.

In assigning a role to amine synthesis as the rate-limiting step in catecholamine recovery, it should be kept in mind that under optimal conditions *in vitro* there is enough tyrosine hydroxylase in the rat adrenal gland to synthesize the entire catecholamine content in 5 hr (15, 29), while recovery of adrenal catecholamines *in vivo* takes 4 days. If synthesis is rate-limiting, then either the tyrosine hydroxylase activity *in vivo* is a small fraction of its total activity *in vitro* or else utilization of the newly synthesized amines is rapid enough to affect the rate of repletion of the catecholamine stores.

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