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ALL-OR-NONE SECRETION OF ADRENAL MEDULLARY STORAGE VESICLE CONTENTS IN THE RAT*

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Abstract—Rats were given insulin (5 i.u./kg) to produce hypoglycemia and a resultant sympathetic discharge and were sacrificed 1, 2, 3 and 4 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 (CA), dopamine β-hydroxylase (DBO; a marker for vesicle membranes) and ATP. One hr after insulin, CA, DBO and ATP in the intact vesicles had fallen to 60 per cent of control, and after 2 hr, to 50 per cent of control. DBO, but not CA or ATP, increased in the empty vesicle fraction. After 3 hr, vesicular CA and ATP had fallen to 35 per cent of controls, while DBO fell only to 50 per cent; DBO remained elevated in the empty vesicle fraction. The parallel loss of CA, DBO and ATP from the intact vesicles during the early time periods, and the increase in particulate DBO in the empty vesicle fraction suggest that secretion from the rat adrenal medulla occurs by an exocytotic release of the total vesicle content with retention of the vesicle membrane, and that release of vesicle contents is all-or-none. The disparity between the loss of DBO and CA after prolonged stimulation has several possible explanations, among which may be the existence of a population of vesicles having DBO, but little or no CA or ATP, or the beginning of vesicle resynthesis. After insulin administration to another group of rats, the incorporation of epinephrine and metaraminol (MA) by isolated adrenal storage vesicles declined. After 1 hr, total adrenal CA had dropped to 68 per cent of control; epinephrine uptake per gland was 72 per cent, and MA uptake was 75 per cent. CA decreased progressively to 22 per cent of control after 4 hr; epinephrine uptake decreased similarly. MA uptake remained at about 70 per cent of control even after 4 hr. The parallel nature of CA depletion and decrease in epinephrine uptake also suggests that secretion from rat adrenal medullary vesicles is exocytotic and all-or-none. The absence of a parallel decrease in MA uptake may mean that MA is taken up into vesicles which do not have a normal CA content ("immature" vesicles). The existence of immature vesicles 3 hr after insulin administration suggests that the rate of vesicle turnover in the rat adrenal medulla is rapid.

THE STORAGE vesicles of the adrenal medulla contain ATP, chromogranins and dopamine β-hydroxylase in addition to catecholamines. Studies of the mechanism of secretion of the vesicles have utilized measurements of all of these parameters. Thus, stimulation of isolated perfused cat adrenals results in secretion of both catecholamines and adenine nucleotides in the molar ratio of 4:1;[1–3] similar results are obtained with bovine adrenals.[4] Stimulation of perfused glands also results in release of chromogranin A and other soluble vesicular proteins, but not of cytoplasmic proteins.[5,6] Release of both catecholamines and chromogranin A from stimulated calf adrenals is also observed *in vivo*.[7]

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These studies indicate that secretion involves the entire soluble contents of the storage vesicle. They do not show, however, whether the entire vesicle is extruded, or whether secretion occurs by exocytosis, wherein the vesicle membrane fuses temporarily with the cell membrane and is left behind subsequent to release of the vesicle contents. To distinguish between these hypotheses, Viveros *et al.*[8–10] measured activities of dopamine β-hydroxylase, an enzyme associated with both the soluble contents of the vesicle and also with the vesicle membrane.[11] Stimulation of rabbit adrenal glands, produced by insulin-induced hypoglycemia, resulted in reductions in both catecholamines and in dopamine β-hydroxylase in the fraction containing empty vesicle membranes. These results are consistent with the view that release occurs by exocytosis, since the soluble contents are lost, and broken vesicle membranes remain. Similar conclusions have been reported in publications from other laboratories.[12–15]

Additional studies have been concerned with whether secretion is all-or-none, i.e. whether the minimum response to stimulation is the release of the entire contents of one vesicle. It was found that, 3 hr after insulin administration in rabbits, there was no change in the ratio of dopamine β-hydroxylase to catecholamines in the intact vesicles remaining in the gland.[10,16] This is consistent with the view that secretion is all-or-none; if the vesicles released only part of their contents, the ratio of dopamine β-hydroxylase to catecholamines would have risen because a large part of the enzyme (associated with the vesicle membrane) would still be present in the partially depleted intact vesicles. However, one difficulty in interpreting these studies resides in the fact that, in most cases, extensive secretion had occurred.[10,16] After extensive stimulation, it would be difficult to establish whether or not all-or-none secretion occurred throughout the stimulation period, since massive secretion would result in total emptying of the storage vesicles. The remaining vesicles might represent those the gland is unable to secrete, either from being refractory to further stimulation, or from the reduced chances of a vesicle's being near enough to a cell membrane to fuse with it to initiate secretion.

In the present study, this problem is approached by studying the intermediate degrees of depletion of adrenal vesicle contents in rats, and the decreased ability of the vesicles to incorporate both catecholamines and non-catecholamines. These data can determine whether secretion is all-or-none as well as exocytotic.

METHODS

Male albino rats of the Sprague–Dawley strain (200–250 g) were fasted 24 hr and given insulin (5 i.u./kg) via a tail vein; animals were sacrificed by decapitation 1, 2, 3 or 4 hr after drug administration. The adrenal glands from each animal were excised, cleaned of fat and connective tissue, and homogenized (glass-to-glass) in 2·5 ml of ice-cold sucrose–Tris [300 mM sucrose containing 25 mM Tris (pH 7) and 0·01 mM iproniazid (to irreversibly inhibit monoamine oxidase)]. The suspension was centrifuged at 800 *g* for 10 min and the supernatant 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 ml of supernatant was added to 1 ml water, homogenized and assayed (fraction B). Another ml of the 800 *g* supernatant was layered over 2·5 ml of 1·6 M sucrose containing 500 units/ml of beef catalase (Sigma) and centrifuged for 2 hr at 140,000 *g* in the No. 40 rotor of the

Beckman model L2 ultracentrifuge. This separates intact vesicles from broken vesicle membranes and from most mitochondrial and lysosomal contaminants.[17] The 0·3 M 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 (CA) and dopamine β-hydroxylase (DBO). The vesicular pellet (fraction E) was resuspended in 2 ml water and homogenized to lyse the vesicles. One ml was removed for the determination of CA and DBO, and the remainder was centrifuged at 26,000 *g* to remove the vesicle membranes; the supernatant of this latter centrifugation was analyzed for ATP. A flow sheet of the procedure appears in Fig. 1.

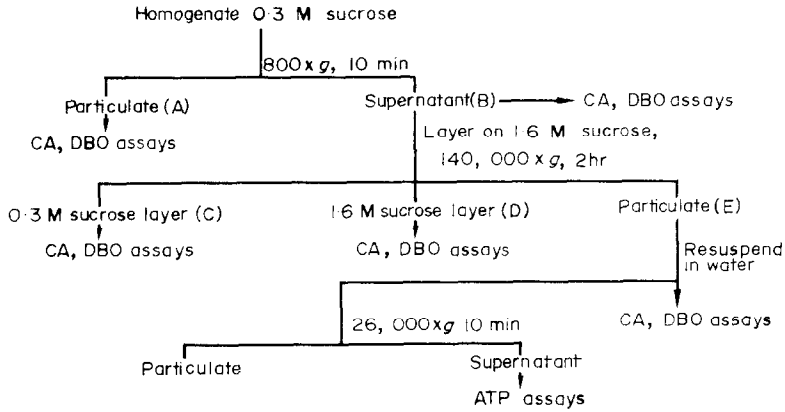


FIG. 1. Fractionation of adrenal homogenates.

Distribution of newly incorporated amines. Adrenal glands from ten rats were homogenized in 33 ml sucrose–Tris and centrifuged 10 min at 800 *g*. Fifteen ml of vesicle suspension was added to 5 ml of sucrose–Tris containing 20 mM ATP, 20 mM Mg^{2+}, 0·4 mM epinephrine and either 3 μCi ^{14}C-epinephrine or 15 μCi ^{3}H-metaraminol plus 0·4 mM metaraminol. The mixtures were incubated 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 sucrose–Tris. Sets of 15, 1-ml aliquots of labeled vesicles were brought to 37° 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 CA and radioactivity. The vesicular pellets were resuspended in 3 ml of 3·5% perchloric acid (to lyse the vesicles), centrifuged 10 min at 26,000 *g* (to remove precipitated proteins), and analyzed for CA and radioactivity. The effluxes of endogenous CA, ^{14}C-epinephrine and ^{3}H-metaraminol were calculated as described previously.[18]

Uptake of radioactive amines. Rats were given insulin and sacrificed as already described. The glands from each animal were homogenized in 2·2 ml sucrose–Tris and an aliquot was widthdrawn for CA analysis. After the suspension was 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 ^{14}C-epinephrine or 5 μCi ^{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 CA 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 CA and radioactivity. Uptake in each sample was calculated as described below:

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

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

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

Assays. Aliquots (0·1 ml) of all catecholamine samples were added to 1·9 ml of 3·5% perchloric acid and centrifuged at 26,000 *g* for 10 min in order to remove precipitated protein. The supernatants were analyzed for CA by the trihydroxyindole method, as described previously,[18] and reported as microgram equivalent of epinephrine.

Radioactive amines were measured by liquid scintillation spectrometry. One ml of each sample was added to 10 ml of a 1:2 mixture of Triton X-100 detergent and toluene (containing fluors PPO and POPOP).*

ATP was analyzed by the luciferin–luciferase method.[19] The contents of a vial of buffered firefly lantern extract was dissolved in 5 ml water; 0·2 ml of enzyme, 0·5 ml water and 0·2 ml of sample were used in each assay. Phosphorescence was determined in a Farrand spectrofluorometer 20 sec after the addition of the ATP-containing sample.

Dopamine β-hydroxylase was assayed using ^{3}H-tyramine as described previously;[20] 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. *para*-Hydroxymercuribenzoate (PMB) was used to inactivate endogenous inhibitors;[21] optimal PMB concentrations were: fraction A, 1 mM; fractions B and C, 0·5 mM; fraction D, 0·025 mM; fraction E, 0.

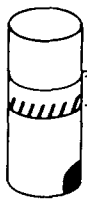
Statistical analyses. Data are reported in terms of control values and percentage of control. Levels of significance are calculated by Student's *t*-test; straight lines are determined by the method of least squares, and paired parameters are evaluated by determination of linear correlation coefficients.[22]

* PPO = 2,5-diphenyloxazole; POPOP = 1,4-*bis*-2-(5-phenyloxazolyl) benzene.

Materials. Epinephrine-7-^{14}C, metaraminol-7-^{3}H, and tyramine-G-^{3}H were obtained from New England Nuclear Corp. Buffered firefly extract was obtained from Worthington Biochemicals, 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 vesicles and vesicle constituents. The distribution of CA and DBO after discontinuous density gradient centrifugation of rat adrenal medullary vesicles is shown in Fig. 2; the addition of catalase to the 1·6 M sucrose was required to maintain enzyme activity. Approximately one-fourth of the intact vesicles were broken during the procedure; almost all of the CA and DBO in the remaining vesicles appeared in the pellet. To evaluate the ability of this procedure to separate intact vesicles from vesicle membranes, a preparation of water-lysed vesicles was also centrifuged in a like manner (Fig. 2). In the lysed preparation, more than 90 per cent of the CA and DBO appeared in the light sucrose layer, and contamination of the pellet with CA and DBO was small. These data indicate that the method is adequate for separation of intact from disrupted storage vesicles. Little or no ATP was found in any fraction except the intact vesicular pellet.



Fraction	Sucrose conc.	Intact Vesicles CA	Intact Vesicles DBO	Lysed Vesicles CA	Lysed Vesicles DBO
C	0.3 M	23	23	91	94
D	1.6 M	7	0	0	3
E	—	70	78	8	3

FIG. 2. Relative distributions of catecholamines and dopamine β-hydroxylase after discontinuous sucrose density centrifugation of homogenates containing intact or lysed rat adrenal storage vesicles.

In addition to demonstrating that fraction E was not contaminated with broken vesicle membranes, it was important to establish that fraction C was not contaminated with intact vesicles. To determine this, the 800 *g* supernatant was centrifuged at 26,000 *g* to precipitate storage vesicles and broken membranes. The CA remaining in the supernatant, a corroborative measure of the fraction of broken vesicles, was 20 per cent, which was close to the value found for CA and DBO in fraction C (23 per cent). This indicated that fraction C was not contaminated with intact vesicles, since the CA and DBO in fraction C would have been higher than the CA in the 26,000 *g* supernatant if contamination occurred.

The subcellular distribution of CA and DBO in control animals is shown in Table 1. Approximately 15 per cent of the total CA and DBO was found in the 800 *g* pellet (fraction A); this probably represented the fraction of cells not broken during homogenization of the gland, and was consistent from preparation to preparation. Recoveries from the discontinuous gradient [(C + D + E)/B] (Table 1) were about 95 per cent for CA and 85 per cent for DBO. Approximately 20–25 per cent of the CA and DBO on the gradient was found in the soluble, 0·3 M sucrose layer; this probably represents vesicles broken during the preparative procedure. Recovery of CA and

TABLE 1. SUBCELLULAR DISTRIBUTION OF CATECHOLAMINES, DOPAMINE β-HYDROXYLASE AND ATP

	Time after insulin (hr)	A*	B	C	D	E	Total (A + B)	No. of animals
Catecholamines (% of control)								
Control (μg/gland)		1·42 ± 0·06	7·26 ± 0·20	1·68 ± 0·29	0·68 ± 0·07	4·56 ± 0·15	8·65 ± 0·25	39
	0	100 ± 4	100 ± 3	100 ± 17	100 ± 10	100 ± 3	100 ± 3	39
	1	58 ± 5	66 ± 3	76 ± 4	79 ± 5	57 ± 4	64 ± 4	18
	2	47 ± 4	50 ± 3	63 ± 4	64 ± 6	45 ± 3	50 ± 3	21
	3	35 ± 4	40 ± 3	61 ± 4	54 ± 6	34 ± 3	39 ± 3	18
Dopamine β-hydroxylase (% of control)								
Control (nmoles/gland/hr)		0·09 ± 0·01	0·63 ± 0·04	0·12 ± 0·02	< 0·02	0·40 ± 0·02	0·71 ± 0·05	16
	0	100 ± 11	100 ± 6	100 ± 17		100 ± 5	100 ± 7	16
	1	87 ± 11	87 ± 8	158 ± 17		63 ± 8	86 ± 8	6
	2	84 ± 12	73 ± 9	144 ± 28		57 ± 6	74 ± 9	5
	3	100 ± 7	85 ± 4	169 ± 28		48 ± 3	87 ± 4	6
ATP (% of control)								
Control (nmoles/gland)						6·7 ± 0·4		23
	0					100 ± 6		23
	1					60 ± 4		18
	2					49 ± 3		15
	3					38 ± 4		14

* A = 800 *g* pellet; B = 800 *g* supernatant; C, D, E = discontinuous sucrose density gradient; C = 0·3 M sucrose layer (broken vesicle membranes); D = 1·6 M sucrose layer; E = vesicular pellet (intact vesicles). All values are given as means ±S.E.

DBO in the purified vesicular fraction (E) averaged 60–65 per cent of the amount placed on the gradient, or about 55 per cent of the whole gland CA and DBO contents. About 80 per cent of the vesicular DBO was membrane-bound, and about 20 per cent was soluble upon lysis of the vesicles. The CA recovery values from the gradient agree with those of Smith and Winkler.[17]

Fraction E contained on the average, 6·7 nmoles ATP/gland and 25 nmoles CA. This gives a molar ratio of CA/ATP of 3·7, which is close to the theoretical value of 4.

Effect of insulin on CA, DBO and ATP distribution. Within 1 hr of insulin administration, catecholamine levels in all subcellular fractions were decreased; fractions A, B and E contained 60 per cent of control values (Table 1). At 2 and 3 hr after insulin, catecholamine levels in these fractions dropped to 50 and 40 per cent of control respectively. Catecholamines in fractions C and D decreased, although to a lesser extent than in the other fractions, but since the combined CA content of these fractions was relatively small, the total CA content also fell to values similar to those for fraction E. Thus, insulin administration led to a progressive depletion of adrenal CA, and the total CA mirrored the decrease in the purified vesicles.

In contrast to CA, there was only a small decrease in total DBO after insulin administration (Table 1). However, there was a marked shift in the subcellular distribution of the enzyme. The DBO in fraction C (vesicle membranes) increased markedly, while at 1 and 2 hr the DBO in the intact vesicle fraction (E) decreased similarly to CA. There was little or no change in the amount of DBO in the 800 *g* pellet (fraction A; Table 1). At 3 hr, there was a significant ($P < 0{\cdot}01$) difference between the degree of CA depletion and that of DBO depletion in the intact vesicles; this difference is emphasized by a scattergram showing individual vesicular levels of CA and DBO (Fig. 3). The least squares line best fitting these points indicates that a significant amount of vesicular DBO would remain even after complete CA depletion, despite the high correlation between CA and DBO in the vesicles ($r = 0{\cdot}89$).

The vesicular ATP levels decreased in a linear fashion with CA levels (Table 1). At no time after insulin was there a significant difference between CA depletion and

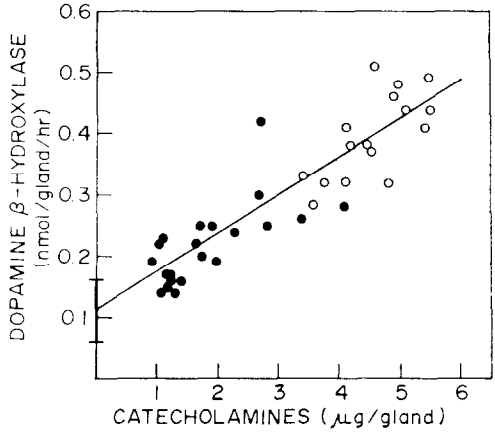


FIG. 3. Scattergram of vesicular dopamine β-hydroxylase (nanomoles per hour per gland) and catecholamines after insulin administration, with least squares line fit to the points. Open circles denote controls; solid circles denote insulin-treated. The vertical bar denotes ± standard error of intercept. The intercept with the ordinate is significantly different from zero ($P < 0{\cdot}05$).

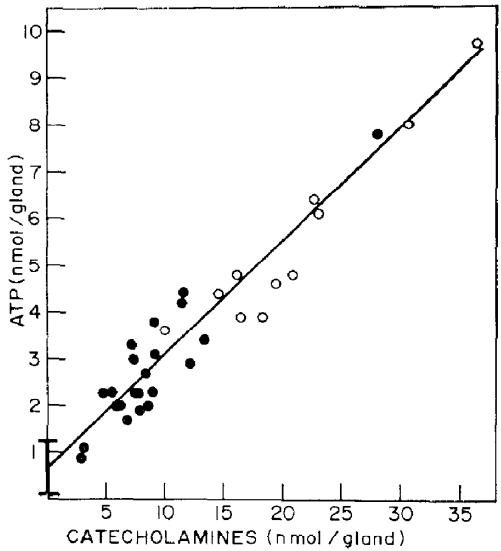


FIG. 4. Scattergram of vesicular ATP and catecholamines after insulin administration, with least squares line fit to the points. Open circles denote controls; solid circles denote insulin-treated. The vertical bar denotes ± standard error of intercept. The intercept with the ordinate is not significantly different from zero ($P > 0{\cdot}05$).

ATP depletion; this was confirmed by examining a scattergram of individual vesicular CA and ATP levels (Fig. 4). In this case, the least squares line intersects the ATP-axis at a point not significantly different from the origin, indicating that little or no

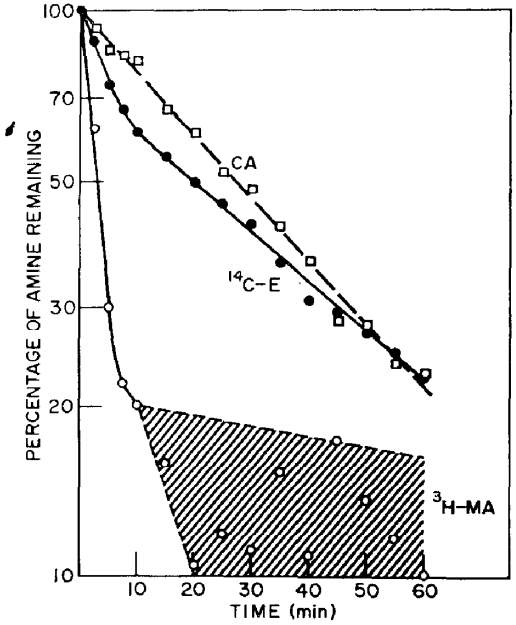


FIG. 5. Efflux of endogenous catecholamines (CA) □- - -□, ^{14}C-epinephrine (^{14}C-E) ●——● and ^{3}H-metaraminol (^{3}H-MA) ○——○, from isolated rat adrenal storage vesicles.

TABLE 2. UPTAKE OF EPINEPHRINE AND METARAMINOL BY A CRUDE PREPARATION OF ADRENAL STORAGE VESICLES*

Time after insulin (hr)	Catecholamines	Epinephrine uptake		Metaraminol uptake		No. of animals
		(per gland)	(per 100 μg CA)	(per gland)	(per 100 μg CA)	
Control	7·81 ± 0·44†	1·45 ± 0·13‡	18·4 ± 1·1§	0·23 ± 0·01‡	2·93 ± 0·10§	16
0	100 ± 6	100 ± 9	100 ± 6	100 ± 4	100 ± 3	16
1	68 ± 5	72 ± 4	105 ± 5	75 ± 5	111 ± 4	6
2	47 ± 5	55 ± 9	115 ± 8	74 ± 7	157 ± 19	4
3	37 ± 3	46 ± 3	122 ± 7	83 ± 7	228 ± 10	6
4	22 ± 2	22 ± 5	93 ± 14	67 ± 7	300 ± 29	6

* All values are given as means ±S.E. All experimental values are expressed as per cent of control.
† Micrograms per gland.
‡ Nanomoles taken up per gland in 30 min.
§ Nanomoles taken up in 30 min/100 μg of catecholamines in vesicles.

vesicular ATP was found unassociated with CA. In addition, the correlation coefficient was higher than for CA and DBO ($r = 0{\cdot}96$). CA divided by ATP (the reciprocal of the slope function in Fig. 4) had a value of slightly more than 4, which is the theoretical molar ratio of CA to ATP within the vesicle.

Distribution of newly incorporated amines. Newly incorporated ^{14}C-epinephrine and ^{3}H-metaraminol exhibited biphasic rates of efflux (Fig. 5). Graphical analysis of these curves[18] indicates a fit to two independent compartments, one with a half-life of about 3 min ("fast" pool), and the other with a longer half-life ("slow" pool). The distributions of epinephrine and metaraminol differed markedly (Fig. 5). Most (> 75 per cent) of the epinephrine was incorporated into the slow pool, while most (> 80 per cent) of the metaraminol was incorporated into the fast pool. The half-life of either amine in the fast pool was about 3 min; the half-life of epinephrine in the slow pool was 33 min, while the half-life of metaraminol in the slow pool was indeterminate because of the low levels of ^{3}H-metaraminol in this pool (Fig. 5). The half-life of endogenous amines was quite similar to that of the slow pool of ^{14}C-epinephrine. These studies indicated that the uptakes into either pool could be studied selectively by using the appropriate amine.

Uptake of amines after insulin administration. These data are reported in terms of uptake per gland and uptake per 100 μg of CA in the vesicles (Table 2). The former parameter measures the number of functional uptake units remaining in the gland, while the latter parameter describes the ability of each vesicle to take up amines relative to the amount of CA already present within the vesicles.

Adrenal vesicles isolated from control animals incorporated 1·5 nmoles epinephrine per gland in 30 min, or slightly more than 3 per cent of the endogenous level (Table 2). After insulin administration, the uptake of ^{14}C-epinephrine per gland decreased in a manner similar to the decrease in CA (Table 2). After 4 hr, only 22 per cent of the CA and uptake remained. The high correlation coefficient between total CA and ^{14}C-epinephrine uptake per gland ($r = 0{\cdot}91$) and the scattergram (Fig. 6) of individual

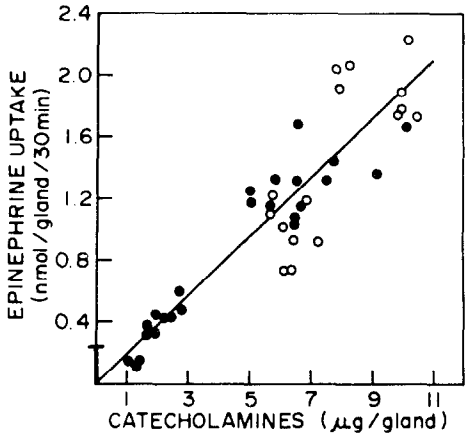


FIG. 6. Scattergram of vesicular epinephrine uptake and total catecholamine content, with least squares line fit to the points. Open circles denote controls, solid circles denote insulin-treated. The vertical bar denotes ± standard error of intercept. The intercept with the ordinate is not significantly different from zero ($P > 0{\cdot}05$).

values indicated a one-to-one relationship between CA and uptake. When the uptake was expressed in nanomoles per 100 μg of CA in the vesicles, there was no difference between insulin-treated and control animals, indicating that uptake per intact vesicle remained unchanged.

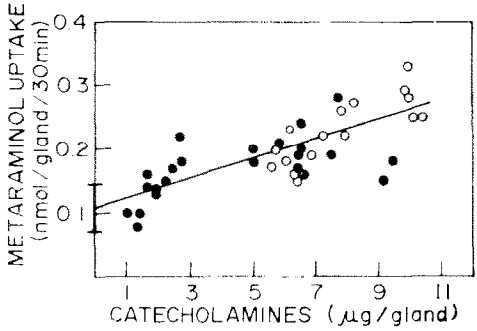


FIG. 7. Scattergram of vesicular metaraminol uptake and total catecholamine content, with least squares line fit to the points. Open circles denote controls, solid circles denote insulin-treated. The vertical bar denotes $\pm$ standard error of intercept. The intercept with the ordinate is significantly different from zero ($P < 0{\cdot}005$).

For ^{3}H-metaraminol uptake per gland vs. CA, there was a lower correlation coefficient than for epinephrine ($r = 0{\cdot}75$), and the scattergram indicated that a substantial proportion of metaraminol uptake would remain, even in the absence of CA (Fig. 7); the least squares line did not pass through the origin (Fig. 7). In addition, there was a high negative correlation between metaraminol uptake per 100 μg of CA and the CA remaining after insulin administration ($r = -0{\cdot}86$). Thus, the decrease in metaraminol uptake per gland was much less than the decrease in CA following insulin, and the ability of the remaining vesicles to take up metaraminol tripled relative to the amount of CA present within the vesicles (Table 2). Consequently, the decrease in uptake per gland for epinephrine (Fig. 6) followed a pattern similar to that of CA and ATP (Fig. 4), while the decrease in metaraminol uptake per gland (Fig. 7) differed from that of CA to a greater extent than did the decrease in vesicular DBO (Fig. 3).

DISCUSSION

If secretion from the adrenal medulla occurs by exocytosis, then the following criteria must be met: (1) Equivalent release of other soluble vesicle constituents, along with catecholamines should occur. (2) Empty vesicle membranes should appear after release of the soluble contents. (3) The ability of the storage vesicles to incorporate catecholamines should decrease parallel to the decrease in catecholamine levels at all stages of depletion; this assumes that the ATP and chromogranins which are released along with CA are responsible for the stability of the amine storage complex. In addition, if all-or-none secretion occurs, (4) the ratio DBO/CA in the remaining vesicles should be the same as in the original population.

After insulin administration, vesicular ATP decreased at the same rate as catecholamines; one ATP molecule was released for every four catecholamine molecules

(Fig. 4). This is consistent with the intravesicular ratio of the two compounds, indicating that other soluble constituents are released at the same rate as catecholamines, thus confirming the first criterion for exocytosis.

Unlike catecholamines, there was only a small decrease in total dopamine β-hydroxylase after insulin administration, although the subcellular distribution shifted markedly. There was little or no change in the amount of dopamine β-hydroxylase found in the 800 *g* pellet (fraction A), which contains the broken cell membranes. This implies that the joining of vesicle to cell membrane which would occur during exocytosis is short-lived, since a long-term attachment would result in increased dopamine β-hydroxylase activity in the 800 *g* pellet. There was a similar decrease of catecholamines and dopamine β-hydroxylase in the fraction containing the intact vesicles, and most of the lost enzyme activity appeared in the fraction containing broken vesicle membranes. Since dopamine β-hydroxylase is associated mostly with the vesicle membrane in rats, this suggests that the release of catecholamines is accompanied by a decrease in the number of intact vesicles, and a consequent increase in the amount of broken vesicle membranes. This confirms criterion 2; the only loss of total dopamine β-hydroxylase should be that portion which is in the soluble fraction, not attached to the vesicle membrane. Viveros *et al.*[8–10] have made similar observations in rabbits.

The equivalent nature of the initial decrease in vesicular CA and DBO also implies that release of vesicle contents occurs by an all-or-none process. If partial release of the contents had occurred, a significantly greater proportion of CA than DBO would be released at all time periods, and the remaining vesicles would have higher ratios of DBO to CA. In the initial period, there was no difference between release of CA and of DBO. In their studies of insulin-induced CA depletion in rabbits, Viveros *et al.*[10] did not consider the possibility that the constant nature of DBO/CA might reflect the fact that severe depletion had caused total vesicle emptying in a non-quantal fashion. The intermediate stages examined in the present study are a better indication that all-or-none release occurs.

There was, however, a statistically significant difference in the degree of depletion of vesicular dopamine β-hydroxylase and of catecholamines at 3 hr (Table 1); the scattergram (Fig. 3) suggests that about 25 per cent of the dopamine β-hydroxylase would remain, even after total catecholamine secretion. These data do not agree with those of Viveros *et al.*[10] Several explanations for this discrepancy are possible. The extra dopamine β-hydroxylase might reflect contamination of the vesicle pellet with vesicle membranes, or it could represent a population of intact vesicles which have dopamine β-hydroxylase but little catecholamines. The former explanation seems unlikely, since contamination of the vesicle pellet with membranes from water-lysed vesicles is only 3 per cent (Fig. 2); the possibility cannot be ruled out, however, that stimulation results in a change in the vesicle membranes such that there is greater contamination of the pellet with empty membranes. Indeed, although the amounts involved are small, the smaller reduction of catecholamines in fraction C of the gradient compared to fraction E (Table 1) after insulin could reflect a change in the vesicles such that they are more fragile. If such a change occurs, then the aggregation of intact vesicles and empty membranes might also be altered.

The discrepancy between the loss of vesicular catecholamines and dopamine β-hydroxylase at 3 hr might better be explained by postulating the existence of

vesicles which have dopamine β-hydroxylase but little or no catecholamines. Viveros *et al.*[10] have observed the formation of such vesicles during recovery from insulin-induced hypoglycemia in rabbits. They have a higher density than the broken vesicle membranes, but a lower density than the normal vesicle population.[10] Presumably, these are vesicles which have structural integrity, but are as yet non-functional, either because of lack of available catecholamines or lack of the ability to take up or store them. Thus, the extra dopamine β-hydroxylase in the present study may indicate the presence of such vesicles in the rat. The experimental conditions do not determine, however, whether this population of vesicles exists in the normal resting state of the animal (as a stage in the synthesis of vesicles) or whether it represents the beginning of resynthesis following massive stimulation. In either case, the results differ from those in rabbits[10] in that vesicles with high DBO to CA ratios appear much sooner in the rat; thus, while DBO/CA is constant 3 hr after insulin administration in rabbits, it is twice normal at the same time period in rats. This suggests that the rate of vesicle synthesis is much higher in rats than in rabbits. Winkler *et al.*[23] have studied carbachol-induced release of radioactively labeled catecholamines and chromogranins from isolated perfused bovine adrenal glands. In these experiments, it was found that the specific activity of the secreted chromogranins was maximal 4 hr after pulse-labeling with radioactive precursors, suggesting that significant amounts of protein incorporation into vesicles can occur in that time period. If the turnover time for vesicular proteins in rats is of the same magnitude, this suggests that the "extra" DBO after 3 hr of stimulation may indeed represent the start of new vesicle synthesis.

Thus, within 3 hr of insulin administration, there are three different sources of DBO activity: the remaining intact vesicles (highest density), the newly synthesized vesicles which lack CA (intermediate density), and the broken vesicle membranes (lowest density).

After insulin administration, the ability of isolated rat adrenal storage vesicles to incorporate epinephrine decreased in a manner identical to the decrease in catecholamines and ATP (Tables 1 and 2, Fig. 6). The loss of the ability to incorporate exogenous amines is to be expected from the all-or-none nature of the release process; the broken vesicles cannot take up catecholamines to any significant extent. The fact that there is little or no change in epinephrine uptake per 100 μg of catecholamines implies that the remaining vesicles have the same ability (relative to their catecholamine contents) to incorporate epinephrine as the original population of vesicles.

The efflux of newly incorporated radioactive amines from isolated rat adrenal vesicles is biphasic, consisting of a rapid initial phase ($t_{1/2} \sim 3$ min) and a slower subsequent phase ($t_{1/2} \sim 35$ min for epinephrine); the latter is similar in characteristics to the efflux of endogenous catecholamines. These data indicate that there are two pools of newly incorporated amines. Metaraminol enters the fast-efflux pool, while epinephrine primarily enters the slow-efflux pool. Similar results have been obtained using isolated bovine adrenal medullary vesicles.[18,24] Centrifugation on either continuous[24] or discontinuous sucrose density gradients indicates that both ^{3}H-metaraminol and ^{14}C-epinephrine are taken up into the storage vesicle fraction of both rat and bovine adrenals, and further studies using the latter preparation suggest that the two pools are independent.

The uptake of metaraminol was considerably less affected after insulin administration than was the uptake of epinephrine. Several explanations for this difference

are possible. First, the discrepancies between metaraminol and epinephrine incorporations and between dopamine β-hydroxylase and catecholamine levels may be related. If non-functional vesicles exist which have dopamine β-hydroxylase, but little catecholamines or ATP, would these vesicles be able to accumulate amines? Previous studies have shown that uptake and binding of amines may be two separate processes,[24] and that amines can penetrate the vesicle membrane without becoming incorporated into the storage complex.[25,26] Thus, intact vesicles which have little ATP may be able to take up amines, but not to store them stably. This would account for the rapid rate of efflux from the fast-releasing pool. Furthermore, the stable binding of amines by the vesicles is structurally selective; catecholamines are bound more stably than non-catecholamines.[24] Epinephrine, therefore, would be incorporated primarily into the stable storage pool. Metaraminol, on the other hand, would enter the rapid-efflux pool preferentially. If the rapid-efflux pool does represent non-functional, "immature" vesicles, then the uptake of metaraminol would be less affected than the uptake of epinephrine after stimulation of the gland. Since these vesicles would have a lower catecholamine content than normal vesicles, the uptake of metaraminol per 100 μg of catecholamines would increase markedly after insulin, as shown in Table 2. At present, a scheme of this type is speculative, but there is a growing body of evidence that non-functional vesicles exist, and that they may be deficient in the ability to store catecholamines.[10]

It should be kept in mind that other explanations of the discrepancy between the incorporations of epinephrine and metaraminol are possible. For example, metaraminol may be taken up by particles other than storage vesicles. However, since purification of labeled vesicles on sucrose density gradients yielded amine incorporations indistinguishable from those obtained with the 800 *g* supernatant fraction, such an explanation seems somewhat less likely.

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