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RELEASE OF CATECHOLAMINES AND DOPAMINE β-HYDROXYLASE FROM THE PERFUSED ADRENAL GLAND OF THE CAT

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SUMMARY

1. Secretion of catecholamines (CA) and dopamine β-hydroxylase (DBH) activity from the perfused cat adrenal gland was studied following splanchnic nerve stimulation or infusion of acetylcholine (ACh).

2. Splanchnic nerve stimulation (30 Hz) or perfusion with a low concentration of ACh (10^{-5} M) caused a marked release of CA in the venous effluent, but release of DBH activity was minimal while a higher concentration of ACh (10^{-4} M) enhanced the release of CA and DBH.

3. The ratio of DBH/CA released in the perfusate by splanchnic nerve stimulation or ACh infusion was only a small fraction of the ratio in the soluble lysate of purified chromaffin vesicles.

4. Following reserpine treatment, adrenal CA levels fell to 25% of the control value in 24 hr, remained depressed on days 2, 3, 4 and 5 at 5% of the control and recovered to 60% of the control value on the 6th day. DBH activity was unchanged from the control value at 24 hr after treatment, then rose as high as 5 times the control on the 5th day and was still twice the control value on the 6th day.

5. CA secretion in response to ACh (10^{-4} M) perfusion was reduced to 30% of the control value on the first day after reserpine treatment, while DBH secretion was unchanged. On the 2nd day, CA secretion was depressed further to 5% of the control and remained at this low level up to 5 days after treatment while DBH secretion was twice the control value at 48 hr and then on days 3, 4 and 5 rose up to 5 times the control value. On the 6th day, secretion of CA recovered to 30% of the control while DBH secretion was now twice the control.

6. Isopycnic sucrose density (discontinuous) gradient centrifugation of vesicles from adrenal glands of control cats, and of cats given reserpine 1 or 2 days previously, indicated that new vesicles or vesicles depleted of CA by reserpine had a lower equilibrium density than the original population of vesicles.

7. These results suggest that the release of CA is quantal in nature, but the release of DBH is not necessarily coupled with it. Release of DBH by ACh from reserpinized glands suggests that the vesicles which were once involved in secretion may be re-used for synthesis and storage of CA.

INTRODUCTION

A large body of evidence supports the view that release of CA from the adrenal medulla occurs by exocytosis. Secretion of CA from the adrenal gland is accompanied by a simultaneous release of ATP (Douglas & Poisner, 1966), chromogranins (Blaschko, Comline, Schneider, Silver & Smith, 1967) and DBH (Viveros, Arqueros & Kirshner, 1968).

Secretion of CA by exocytosis implies that the ratio of CA to ATP or soluble proteins in perfusates should be close to that in the whole adrenal gland. Douglas & Poisner (1966) demonstrated a proportional release of CA and ATP from the perfused cat adrenal gland. Secretion of CA from the bovine adrenal gland in response to splanchnic nerve stimulation or to an injection of ACh was also accompanied by release of chromogranin and DBH; the ratios of CA to these proteins were found to be very similar to their respective ratios in the soluble lysate of chromaffin vesicles (Schneider, Smith & Winkler, 1967; Banks & Helle, 1965; Blaschko *et al.* 1967; Viveros *et al.* 1968). Comparable studies have not been reported, so far, in laboratory animals. In order to get some further insight into the mechanism of excitation–secretion coupling, we have studied the release of CA and DBH from perfused cat adrenal glands. We were able to show that DBH was discharged in the perfusate when secretion of CA was evoked either by splanchnic nerve stimulation or injection of ACh, but the ratio of DBH to CA released in the perfusate was only a small fraction of that found in the soluble lysates of chromaffin vesicles. Additional studies in reserpinized cats showed that ACh was able to induce release of DBH from glands containing almost no CA, suggesting thereby an independence of release of DBH and CA. A preliminary report of some of these findings has already appeared (Garcia, Kirpekar & Dixon, 1973; Kirpekar, Garcia & Dixon, 1974).

METHODS

Perfusion of cat adrenal gland in situ

Cats were anaesthetized with ether, followed by chloralose (40–60 mg/kg, I.V.). The abdomen was opened by a mid line incision, the stomach, intestines, spleen and right adrenal gland were removed, and the left splanchnic nerve was prepared for stimulation. The left adrenal gland was perfused with a modified Krebs solution by placing a cannula into the abdominal aorta, according to the procedure of Douglas

& Rubin (1961). The perfusate was collected through a cannula inserted into the adrenolumbar vein via the renal vein. The gland was perfused at a flow rate of about 1 ml./min using a perfusion pump.

Perfusion of isolated cat adrenal gland

In some experiments, the gland was removed from the cat after insertion of a cannula into the adrenolumbar vein and perfused in a retrograde direction through the adrenolumbar vein. The gland was placed on a glass funnel, and the surface of the gland was then covered with minute incisions made with a hypodermic needle. The perfusion rate was also about 1 ml./min.

Perfusion of bovine adrenal gland

Adrenal glands were obtained about 30 min after the animals were killed, and were kept on ice until retrograde perfusion was started through a cannula inserted in the adrenal vein. The perfusion technique was essentially similar to that of the isolated cat adrenal gland, but the flow rate was about 4 ml./min.

Perfusion media

All glands were perfused at room temperature with oxygenated-modified Krebs solution which contained 0·25% bovine serum albumin but no bicarbonate. The pH of the solution was adjusted to 7·4 with Tris buffer (1 mM). In some experiments calcium was removed from the solution. After 1 hr of initial perfusion, a 10-min sample was collected to determine the resting secretion of CA and DBH. Secretion was evoked by stimulating the splanchnic nerves at 30 Hz (15 V, 1-msec duration) for 10 min, or by perfusion with a modified Krebs solution containing 10^{-5} or 10^{-4} M-ACh for 10 min. After the stimulation the perfusion fluid was changed back to modified Krebs solution as before. Samples were collected for the duration of the stimulation period, plus four additional post-stimulation samples of 10 min each. Resting levels of CA and DBH activity of pre-stimulation samples were always subtracted from stimulation samples in order to obtain net release of CA and DBH.

Subcellular fractionation of adrenal glands

One cat adrenal gland (normal or reserpinized) or 100 mg of bovine adrenal medulla was chopped and homogenized (Teflon to glass) in 2·5 ml of 0·3 M sucrose solution containing 25 mM Tris, pH 7, and 0·01 mM iproniazid. One or both of the following fractionation procedures were then employed. Procedure (1): the homogenate was centrifuged at 800 *g* for 10 min and the supernatant decanted and centrifuged at 26,000 *g* for 20 min; the sediment from the 26,000 *g* centrifugation, containing the chromaffin vesicles, was rehomogenized (glass to glass) in cold distilled water and centrifuged again at 26,000 *g* for 20 min. The final supernatant contained the soluble DBH, and the pellet the particulate DBH. CA content and DBH activity of all fractions were estimated as described below. Procedure (2): the homogenate was centrifuged at 800 *g* for 10 min and the supernatant decanted. The sediment was rehomogenized in 5 ml. ice-cold distilled water and assayed for CA and DBH activity (fraction *A*, Fig. 1). One ml. of the 800 *g* supernatant (fraction *B*) was layered over 2·5 ml. 1·6 M sucrose containing 25 mM Tris, pH 7, and 500 u./ml. beef catalase in order to preserve DBH activity, and then centrifuged at 100,000 *g* for 2 hr. The 0·3 M sucrose layer plus the 0·3–1·6 M sucrose interface (fraction *C*), and the 1·6 M sucrose layer (fraction *D*) and the pellet (fraction *E*), were each diluted to 5 ml, with water and assayed for CA and DBH activity. In some experiments fraction *E*, which contains the chromaffin vesicles in a highly pure form

(Smith & Winkler, 1967), was rehomogenized (glass to glass) in cold water and centrifuged at 26,000 *g* for 20 min. The supernatant contained the soluble DBH and the pellet (resuspended in 5 ml. water) contained the particulate DBH.

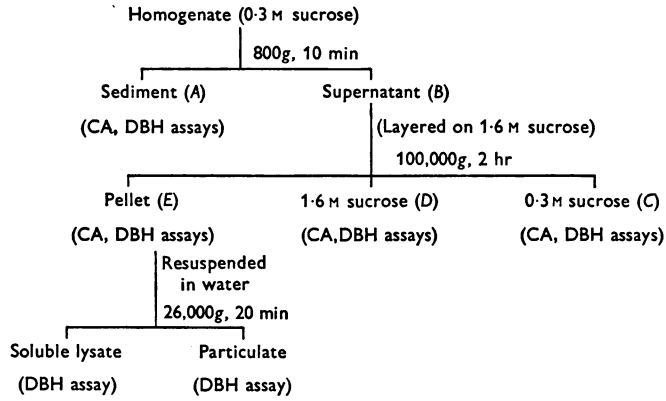

Fig. 1. Subcellular fractionation of adrenal gland (procedure 2). See text for details.

DBH assay

In order to assay total DBH activity, one cat adrenal gland was homogenized in 10 ml. 0·005 M Tris buffer, pH 6·8, containing 0·1 % Triton X-100. The homogenate was centrifuged at 26,000 *g* for 10 min and the enzymic activity of the supernatant assayed. DBH activity of perfusates (0·2 ml.), tissues, and different subcellular fractions (0·01–0·1 ml.) was assayed according to the procedure described by Goldstein, Freedman & Bonnay (1971). The enzyme assay involves a two-step coupled reaction. In the first step of this procedure tyramine is converted to octopamine by DBH at pH 5·5, and in the second step octopamine is N-methylated by S-adenosyl methionine containing tritium in the methyl group in the presence of phenylethanolamine N-methyl transferase (PNMT). DBH activity is determined by analysing the radioactivity incorporated in N-methyloctopamine. In the present work 10 μmole of N-ethylmaleimide (NEM) were used to inactivate endogenous inhibitors of DBH, and 10 μmole of dithiothreitol (Cleland's reagent) were used in the second step to neutralize excess DBH. Incubation time for the first step of the reaction was 30 min for tissue samples and 2 hr for the perfusates. The reaction was linear up to 2·5 hr. The incubation for the second step was 30 min. All incubations were at 37° C. A boiled sample (5 min) served as a blank (500–700 c.p.m.). Tissue activities were at least 3–4 times that of the blank; perfusate activities were from as little as 300 c.p.m. over the blank (e.g. in the case of resting release) to several times the blank. In order to correct for any variation in endogenous inhibitors or activators of the enzyme in each sample (tissue and perfusate), the activity of a fixed quantity of partially purified bovine adrenal DBH (Brimijoin, 1972) was determined, both in the presence and absence of the sample. Recoveries of added DBH activity in the presence of the samples varied between 80 and 100 %, and all sample DBH activities were routinely corrected for recovery. An internal standard of octopamine was always included in each assay in order to convert c.p.m. to units of activity. DBH activity is expressed as nmoles/hr octopamine formed from tyramine.

CA assay

Aliquots of tissue homogenates, subcellular fractions or perfusates were acidified with an equal volume of 0·8 N perchloric acid and centrifuged to remove proteins. Supernatants were then appropriately diluted, and CA contents were assayed in the supernatant as noradrenaline equivalents according to the procedure of Anton & Sayre (1962), without further purification on alumina. CA values are expressed in μg.

Reserpine treatment

Cats were injected I.P. with reserpine 10 mg/kg (Serpasil, Ciba) 1–6 days before the experiment. Cats were very sick after reserpine, and almost 50 % of the animals died.

RESULTS

Relative proportion of soluble and particulate DBH in chromaffin vesicles of the cat and bovine adrenal gland

Secretion of CA by exocytosis implies that the ratio of CA to soluble proteins in perfusates should be close to that in the whole gland. It is therefore important to determine precisely the soluble form of DBH in the chromaffin vesicles, since only the soluble form of this enzyme is supposed to be released during secretion. Using the discontinuous gradient fractionation procedure of Smith & Winkler (1967), a highly purified preparation of chromaffin vesicles was obtained (fraction *E* in Table 4), and osmotic lysis of these vesicles gave an accurate measure of the proportion of 'soluble' DBH to that associated with the vesicular membrane. In the case of the cat adrenal, only 30 % of the DBH present in the vesicular fraction (fraction *E*) was solubilized by osmotic shock, whereas most of the CA was released. The ratio of soluble DBH to CA was $2{\cdot}12 \pm 0{\cdot}53$ (Table 1). In the case of the bovine adrenal gland, of the total DBH activity 43 % was present in the soluble form, and the ratio of soluble DBH to CA was 19·4.

Release of CA and DBH from the cat adrenal by splanchnic nerve stimulation

After 1 hr of initial perfusion of the gland, resting secretion of CA and DBH activity was always low, usually less than 1 μg CA and about 0·25 n-mole/hr of DBH activity per 10-min collection period. In four experiments, splanchnic nerve stimulation (30 Hz) released $5{\cdot}72 \pm 0{\cdot}96$ μg CA, most of which appeared in the venous effluent collected during the 10-min stimulation period (Fig. 2). The net DBH activity released was only $0{\cdot}39 \pm 0{\cdot}15$ n-mole/hr, and most of it appeared slowly in the four 10-min post-stimulation collection periods, while very little activity was detected in the perfusate during the period of nerve stimulation.

In order to ascertain whether any loss in enzymic activity occurred

during its passage through the gland, a partially purified preparation of bovine adrenal DBH was perfused through the cat adrenal, and the enzyme activity was measured in the venous effluent. Recovery of DBH was about 80–100% in these experiments, thereby suggesting that the gland did not inactivate DBH during its passage through the tissue.

TABLE 1. CA and DBH activity in perfusates of adrenal glands after stimulation of the splanchnic nerve or infusion of acetylcholine (ACh). Ratios of DBH to CA in perfusates and adrenals are also shown. For details see text. n = number of experiments. Values are means ± S.E. of mean

			Perfusates			Adrenal soluble DBH/CA in lysates of chromaffin granules
	n	Secretory stimulus	CA (μg)	DBH (n-mole/hr)	DBH/CA	
Cat	5	30 Hz	5·72 ± 0·96	0·39 ± 0·15	0·09 ± 0·04	
	5	ACh(10^{-5} M)	6·91 ± 1·77	1·55 ± 0·72	0·19 ± 0·06	2·12 ± 0·53
	6	ACh(10^{-4} M)	29·54 ± 7·06	6·53 ± 3·04	0·19 ± 0·04	(n = 6)
Bovine	1	ACh(10^{-4} M)	195·5	1,777	9·10	19·4 (n = 2)

Release of CA and DBH from the cat adrenal by ACh

Since ACh is a powerful stimulant of adrenal medullary secretion, secretion was evoked by perfusing the gland with different concentrations of this agent. Fig. 3 shows that perfusion of ACh (10^{-5} M) evoked a secretory response which was very similar to that of splanchnic nerve stimulation. In five experiments, net release of CA and DBH activity evoked by a 10-min perfusion with ACh amounted to 6·91 ± 1·77 μg and 1·55 ± 0·72 n-mole/hr, respectively. For equivalent amounts of CA released, DBH activity appeared to be somewhat higher when secretion was evoked with ACh than with splanchnic nerve stimulation. A higher concentration of ACh (10^{-4} M) markedly increased the secretion of CA and DBH activity to 29·54 ± 7·06 μg and 6·53 ± 3·04 n-mole/hr, respectively, in six experiments (Fig. 4). In one of these experiments secretion of CA amounted to 62·57 μg, and DBH activity to 21·63 n-mole/hr, but the ratio of DBH activity to CA in the perfusate was quite similar to that obtained in previous experiments. DBH secretion lagged behind CA release considerably when secretion was evoked either with low or high concentrations of ACh.

Release of CA and DBH from the bovine adrenal medulla

Viveros *et al.* (1968) demonstrated a proportional release of CA and DBH from the isolated perfused cow adrenal gland when release was

induced by ACh. We carried out one experiment with this preparation. ACh (10^{-4} M) infusion for 10 min released massive amounts of CA (195 μg) and DBH activity (1777 n-mole/hr) in the perfusate.

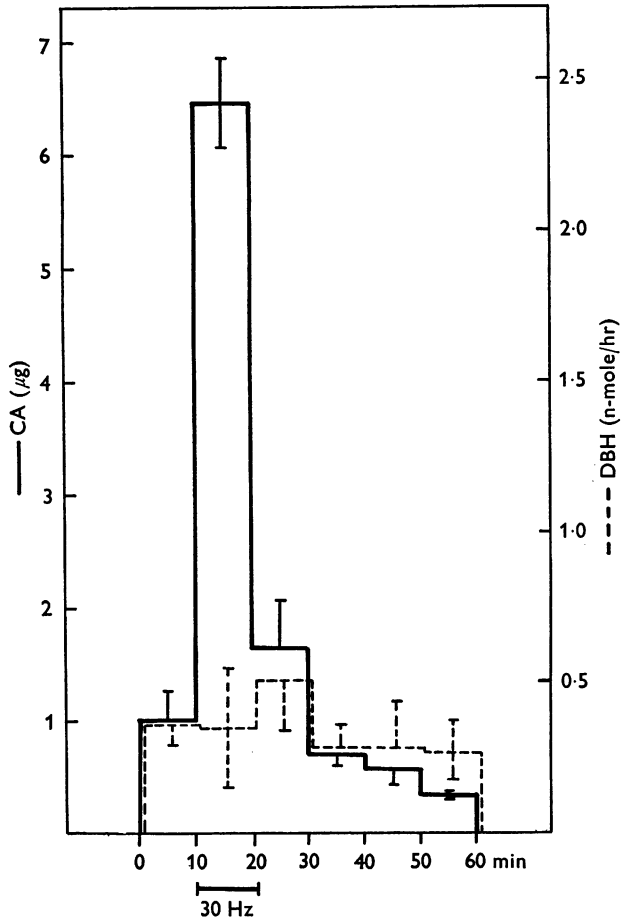

Fig. 2. Release of CA and DBH from perfused cat adrenal glands upon stimulation of the splanchnic nerve at 30 Hz for 10 min. Each bar represents a 10-min collection period. The continuous horizontal line represents the period of stimulation of the splanchnic nerve. Vertical lines are the S.E. of the means (n = 4).

Release of CA and DBH from cat adrenals perfused through the adrenal vein

The perfusion technique for the cat adrenal gland and that for the bovine adrenal gland differed considerably, since the bovine adrenal was perfused in a retrograde manner through the adrenal vein. Therefore, it was of interest to study secretion of CA and DBH from a cat

adrenal gland perfused *in vitro* in a retrograde manner through the adrenal vein, as was done for the bovine adrenal. In three experiments stimulation of the gland with ACh (10^{-4} M) for 10 min caused secretion of 8·81 ± 1·69 μg CA, and 3·80 ± 1·45 n-mole/hr of DBH activity. Both values were lower in comparison with those obtained from glands perfused through the adrenal artery *in situ* (see above).

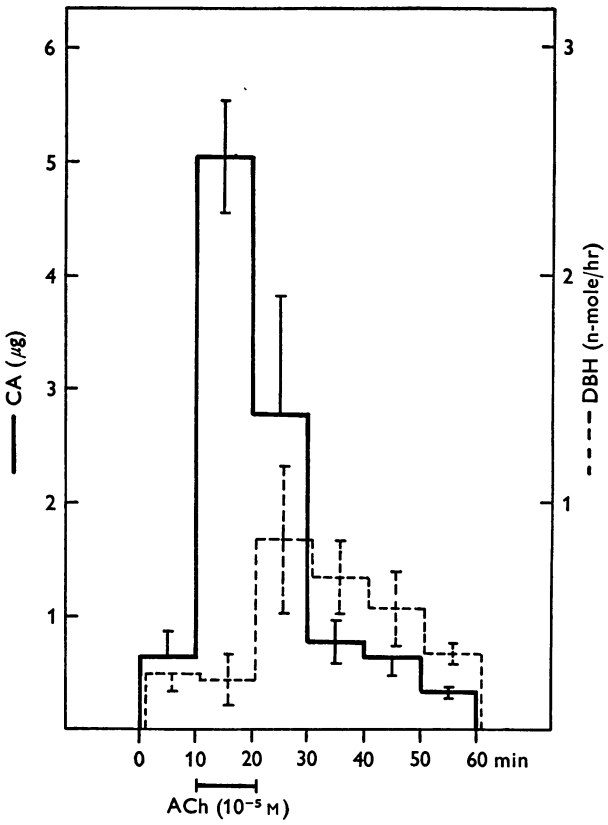

Fig. 3. Release of CA and DBH from perfused cat adrenal glands upon stimulation with a low concentration of acetylcholine (10^{-5} M) for 10 min. Each bar represents a 10-min collection period. The continuous horizontal line represents the time during which the glands were perfused with acetylcholine. The vertical lines are the S.E. of the means (n = 5).

Comparison of ratios of soluble DBH to CA in perfusates and in chromaffin vesicles of cat and bovine adrenal glands

Table 1 compares the ratios of CA and DBH activity secreted in the venous perfusate during splanchnic nerve stimulation or ACh infusion with those in the lysates of purified chromaffin vesicles. The values for

evoked secretion have been calculated by adding CA or DBH activity found in the perfusate after subtracting from each sample the resting levels of both CA and DBH. It can be seen that during splanchnic nerve stimulation the DBH/CA ratio remained very low (0·09); when the gland

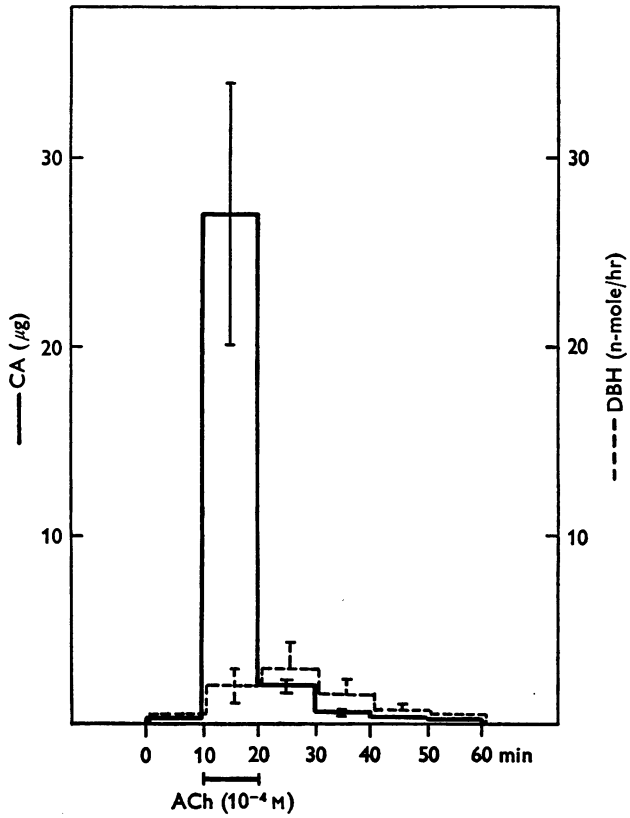

Fig. 4. Release of CA and DBH from perfused cat adrenal glands upon stimulation with a high concentration of acetylcholine (10^{-4} M) for 10 min. Each bar represents a 10-min collection period. The continuous horizontal line represents the time during which the glands were perfused with acetylcholine. The vertical lines are the S.E. of the means (n = 6).

was stimulated with the lower concentration of ACh (10^{-5} M), the DBH/CA ratio was only slightly higher (0·19) than with nerve stimulation. Even though ACh (10^{-4} M) evoked an almost 5-times-larger secretion of CA, the ratio did not change because DBH secretion also increased. The DBH/CA ratio in the perfusate of the bovine adrenal after ACh (10^{-4} M) perfusion was 9·1. Thus, the ratio DBH/CA in lysates of chromaffin vesicles of the cat adrenal gland was about 20 times greater than the ratio

found in perfusates of the nerve-stimulated adrenal, and 10 times greater than the ratio found in perfusates of adrenals stimulated with ACh. However, the DBH/CA ratio of soluble lysates of chromaffin vesicles from the bovine adrenal gland was only two-fold greater than the ratio found in perfusates of the ACh-stimulated gland. These results on the bovine adrenal gland seem to agree fairly well with those of Viveros *et al.* (1968), in spite of the fact that our tissue ratios are twice as high as theirs.

TABLE 2. CA and DBH in normal and reserpinized cat adrenal glands and in their perfusates after stimulation with acetylcholine (10^{-4} M) for 10 min. n = number of experiments. Values are means ± S.E. of mean

Time after reserpine (days)	n	CA		DBH activity	
		Perfusate (μg)	Adrenal (μg/gland)	Perfusate (n-mole/hr)	Adrenal n-mole/hr.gland
0	6	29·54 ± 7·06	106·70 ± 6·80	6·53 ± 3·04	995 ± 98
1	3	9·40 ± 4·00	27·00 ± 14·10	7·16 ± 1·67	1070 ± 43
2	4	1·67 ± 0·46	5·80 ± 1·80	14·75 ± 4·20	3304 ± 605
3	3	0·82 ± 0·36	7·13 ± 2·97	52·28 ± 19·01	5221 ± 1267
4	2	0·27	4·79	33·00	3922
5	3	0·38 ± 0·15	3·50 ± 1·06	40·81 ± 18·80	6601 ± 970
6	3	10·02 ± 5·45	65·30 ± 30·70	14·75 ± 4·20	1977 ± 261

Effect of reserpine treatment on CA concentrations and DBH activity of cat adrenal glands

The effect of reserpine on CA concentrations and DBH activity in cat adrenal glands was determined at various time intervals up to 6 days (Table 2). After a single injection of reserpine, CA concentrations were reduced to 25% of the control value in 24 hr, representing a drop from 107 μg/gland in controls to 27 μg/gland in reserpine-treated cats. Adrenal CA concentrations fell further, to 5% of the control value, in the following 3–4 days, and recovered partially, to 60% of the control value, on the 6th day after reserpine injection. Twenty-four hours after reserpine injection, DBH activity was not significantly different from the control level (1070 ± 43 n-mole/hr.gland, as compared to a control value of 995 ± 98 n-mole/hr.gland). However, at 48 and 72 hr after reserpine, the DBH activities dramatically rose to 3304 ± 605 and 5221 ± 1267 n-mole/hr.gland, respectively. On the 6th day the enzyme activity fell from the high level but was still about two-fold greater than the control value.

Release of CA and DBH from adrenals of cats treated with reserpine

Table 2 shows the values for the release of CA and DBH activity from control glands as well as glands from reserpine-treated cats following

stimulation with ACh (10^{-4} M) for 10 min. Even though ACh-evoked secretion of CA one day after reserpine injection was markedly depressed from the control value of $29{\cdot}54 \pm 7{\cdot}06$ μg to $9{\cdot}4 \pm 4{\cdot}0$ μg, the DBH activity

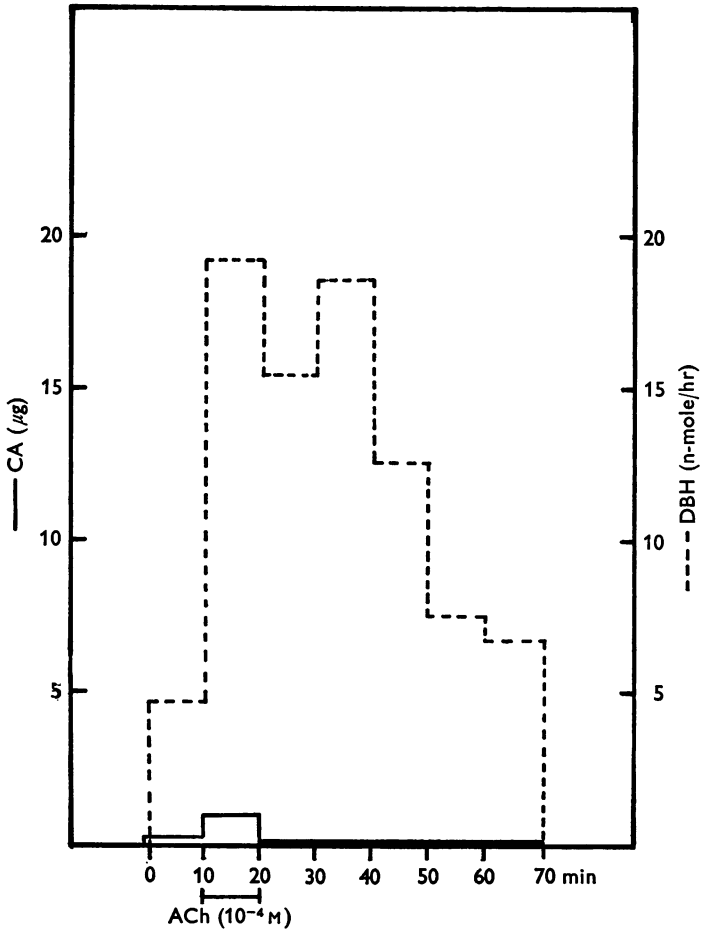

Fig. 5. Release of CA and DBH by ACh (10^{-4} M) from the perfused adrenal gland of a cat pre-treated with reserpine (10 mg/kg) 3 days previously. Each bar represents a 10-min collection period. The horizontal line represents the time during which the gland was perfused with acetylcholine. CA and DBH activity of the gland were 7·13 μg and 5221 n-mole/hr, respectively.

secreted in the perfusate remained unchanged from the release from control glands (7·16 n-mole/hr as compared to 6·53 n-mole/hr for controls). After 2 and 3 days, CA release was further lowered to $1{\cdot}67 \pm 4{\cdot}20$ and $0{\cdot}82 \pm 0{\cdot}36$ μg, respectively, but DBH release increased dramatically to

14·75 ± 4·20 and 52·28 ± 19·01 n-mole/hr. After 6 days, CA secretion was partially restored to 10·02 ± 5·45 μg, representing a recovery of about 30 % of the control output. At the same time, DBH release was reduced but still remained about two-fold greater than the release from normal glands.

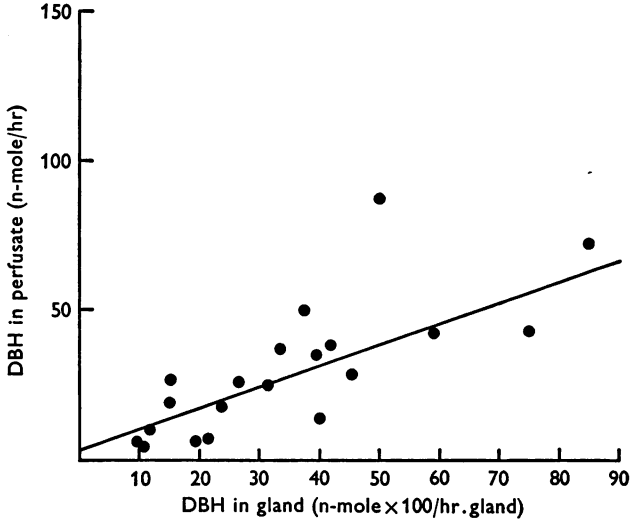

Fig. 6. Scattergram showing relationship between adrenal DBH activity (n-mole/hr.gland) and its release (n-mole/hr) upon stimulation with ACh (10^{-4} M) from normal and reserpinized glands. Correlation coefficient was 0·75 ($P < 0·001$).

The time course of release of DBH by ACh was somewhat different in glands of reserpine-treated cats. In normal glands DBH release lagged behind CA release and occurred only slowly during post-stimulation collection periods. Fig. 5 shows an experiment in which secretion of DBH was evoked by ACh 3 days after reserpine injection. At this time, DBH activity of the gland was increased five-fold over the control value, and DBH release by ACh was also proportionately increased. DBH secretion now appeared in the highest quantity during the stimulation period, remained high for the next two or three collection periods, and then returned to normal resting levels. It should also be pointed out that the resting secretion of DBH was about 10–15 times greater than that obtained from control glands.

Fig. 6 shows the relationship between endogenous levels of DBH and its release by ACh in reserpine pre-treated cats. It can be seen from the regression line that there is a positive correlation between the amount of enzyme present in the gland and that secreted upon stimulation with

ACh ($P < 0{\cdot}001$). Resting secretion also appeared to be directly related to the endogenous levels of DBH.

Effect of calcium on the secretion of CA and DBH during stimulation with ACh

Since calcium is involved in the secretory process of CA from the adrenal medulla, experiments were carried out to determine whether secretion of DBH by ACh, though small, was also dependent on the presence of calcium. In three untreated cats, secretion of CA and DBH from adrenal glands in response to ACh (10^{-4} M) was almost completely blocked when glands were perfused with calcium-free solution. In three cats pre-treated with reserpine 2 days previously, the DBH output in response to ACh (10^{-4} M) infusion was suppressed in the absence of calcium, as in control glands.

TABLE 3. Subcellular distribution of CA and DBH in normal and reserpinized cat adrenal glands, using procedure 1 (see Methods). All values, except the total, are given in percentage of total CA content and DBH activities of the gland. Values are means ± S.E. of mean. n = number of experiments

			Distribution of CA in % of total			
					Granular fraction (26,000 *g* pellet)	
Time after reserpine (days)	n	CA Total (μg/gland)	800 *g* pellet	26,000 *g* supernatant	Lysate	Particulate
0	3	115·4 ± 27·60	25·6 ± 1·4	34·9 ± 0·6	33·1 ± 1·8	6·3 ± 0·3
1	2	39·20	20·1	40·9	33·1	5·8
2	2	10·95	20·0	40·1	33·8	6·0
3	3	10·69 ± 3·88	19·1 ± 2·5	41·3 ± 3·1	33·5 ± 1·2	6·0 ± 1·2
			Distribution of DBH activity in % of total			
					Granular fraction (26,000 *g* pellet)	
Time after reserpine (days)	n	DBH Total (n-mole/ hr.gland)	800 *g* pellet	26,000 *g* supernatant	Lysate	Particulate
0	3	1108 ± 173	21·0 ± 2·5	35·4 ± 2·2	11·8 ± 2·9	31·9 ± 1·6
1	2	1513	23·3	30·3	8·5	37·7
2	2	2290	23·8	33·8	12·8	29·5
3	3	4319 ± 3·2	24·1 ± 3·2	32·8 ± 1·1	13·2 ± 1·1	29·8 ± 1·0

CA content and DBH activity of subcellular fractions of adrenal glands from normal and reserpinized cats

Since DBH activity was greatly enhanced after reserpine treatment, it was of interest to study the subcellular distribution of the enzyme after reserpine administration. Table 3 summarizes the CA content, DBH activity and percentage distribution of different fractions of adrenal

glands fractionated according to procedure 1 (see Methods). In control glands 26% of CA was found in the 800 *g* pellet, 35% in the 26,000 *g* supernatant, and 40% in the 26,000 *g* pellet. DBH distribution followed a very similar pattern. From the total DBH activity of the granular fraction, about one third was solubilized by water osmotic shock, and the remainder appeared to be bound to the particulate membrane fraction.

Twenty-four hours after reserpine injection, CA was decreased by about 70% but the relative distribution in different fractions was unchanged. DBH levels were slightly higher, with no change in relative distribution between fractions. Two and three days after reserpine treatment, CA was markedly reduced but the relative distribution did not appreciably change. DBH activity markedly increased in all fractions, but the relative distribution of the enzyme was similar to that of control glands. The proportion of soluble to particulate DBH in the granular fraction of reserpinized glands was also similar to that found in normal adrenal glands. It was interesting to note that 3 days after reserpine treatment, when the DBH activity was increased by fourfold, the proportion of soluble to particulate DBH in the vesicular fraction did not appreciably change.

Table 4 shows the levels of CA and DBH in different subcellular fractions of normal and reserpinized adrenal glands fractionated according to procedure 2 (see Methods). Of particular interest is the relative distribution of CA and DBH in fraction *C* and fraction *E* which presumably contained intact chromaffin vesicles. About 70% of the CA content of normal adrenals was recovered in these two fractions; fraction *E* accounted for nearly 40% of total CA. DBH was about equally distributed in the two fractions. After treatment with reserpine, distribution of CA, and especially of DBH in fractions *C* and *E*, was dramatically changed. One day after reserpine, CA were markedly reduced without any change in DBH activity. Because of the marked depletion of CA, it was more meaningful to compare the relative distribution of DBH activity rather than CA in different subcellular fractions of the reserpinized gland with that of the normal gland. One day after reserpine neither the DBH activity of the gland nor the relative distribution of soluble to particulate DBH was significantly different from the untreated control glands (see Table 3). However, DBH activity of fraction *C* increased, while that of fraction *E* decreased. Instead of the ratio of the two fractions *E* and *C* being one, as in the control glands, it was now about four. Assuming that the DBH activity is associated with storage vesicles, we suggest that the vesicles which lost their CA by reserpine action and became lighter now appeared in the less dense fraction *C* rather than in the more dense fraction *E*. The ratio of DBH in fraction *C* to that in fraction *E* increased further on day 2 of reserpine injection. Fraction *C* contained most of the DBH

TABLE 4. Subcellular distribution of CA and DBH in adrenal glands of normal and reserpine-treated cats, using procedure 2 (see Methods). A = 800 g pellet; B = 800 g supernatant ($C+D+E$); C, D, E = discontinuous sucrose density gradient (0·3 M sucrose, 1·6 M sucrose and intact chromaffin granules, respectively). Numbers in parentheses denote percentage distribution. n = number of experiments. Values are given as means ± S.E. of mean

Time after reserpine (days)	n	CA (μg) A	$B(C+D+E)$	C	D	E	Total ($A+B$)
0	4	27·31 ± 2·78 (23·2)	90·46 ± 8·44 (76·8)	36·70 ± 5·36 (31·2)	6·34 ± 1·03 (5·4)	47·42 ± 7·48 (40·3)	117·77 ± 11·02 (100)
1	4	9·33 ± 3·18 (32)	19·83 ± 7·18 (68)	12·72 ± 4·81 (43·6)	1·58 ± 0·56 (5·4)	5·53 ± 1·95 (19)	29·17 ± 9·94 (100)
2	3	1·29 ± 0·22 (35·1)	2·39 ± 0·50 (64·9)	1·80 ± 0·33 (48·9)	0·32 ± 0·14 (8·7)	0·27 ± 0·15 (7·3)	3·68 ± 0·71 (100)
		DBH activity (n-mole/hr)					
0	4	94·7 ± 9·8 (13·8)	590·2 ± 87·3 (86·2)	260·2 ± 56·4 (38)	69·6 ± 8·9 (10·2)	260·3 ± 8·9 (38)	747·5 ± 73·7 (100)
1	4	167 ± 9·3 (27·1)	448·5 ± 36·2 (72·9)	309·3 ± 23·1 (50·2)	55·1 ± 4·3 (9·0)	84·1 ± 15·8 (13·7)	615·6 ± 44·2 (100)
2	3	353 ± 13·9 (29·5)	844·2 ± 41·3 (70·5)	603·4 ± 99 (50·14)	154 ± 28·9 (12·9)	87·1 ± 29·9 (7·3)	1197 ± 53 (100)

activity, which was about seven-fold greater than the activity found in fraction *E*.

The fractionation procedure utilizing the discontinuous sucrose gradient gave about 40 % less recovery of total DBH activity. We are unable to give any explanation for this loss.

DISCUSSION

Stimulation of the perfused cat adrenal gland via the splanchnic nerve, or by perfusion of ACh, results in an enhanced release of CA into the venous effluent, but a barely detectable increase in DBH activity. A comparison of the release of CA and DBH by splanchnic nerve stimulation versus perfusion of a low dose of ACh revealed that CA secretion was similar in both instances, while DBH release was higher following perfusion of ACh. The minimal amounts of DBH released in the perfusate following splanchnic nerve stimulation, which is the normal physiological stimulus *in vivo*, raise some doubt as to any relationship between the DBH activity released and the secretion of CA.

In addition, CA are released during the stimulation period and then return to the resting secretion level following termination of stimulation, while DBH levels are unaltered during the stimulation period but increase above the resting level for the 40 min following the end of stimulation. Thus there is a temporal lag in the release of DBH compared to that of CA.

Perfusion of the cat adrenal with a higher concentration of ACh (10^{-4} M) resulted in the release of massive amounts of CA, but only small amounts of DBH. Lowering the calcium concentration of the perfusion solution abolished release of both CA and DBH activity. Douglas & Rubin (1961, 1963) found that omission of calcium ions from the perfusion solution completely inhibited release of CA by ACh from the perfused cat adrenal gland. Therefore the release of DBH, like the release of CA, in response to ACh was dependent on calcium in the perfusion solution.

If the DBH activity released in the perfusate along with CA is representative of the soluble protein component within the vesicle, then the ratio of DBH to CA in the perfusate following stimulation with ACh should be the same as in soluble lysates of chromaffin vesicles isolated from the gland. After perfusion with a higher concentration of ACh, the ratio of DBH to CA released in the perfusate was 0·19, which was only one-tenth of that in lysates of chromaffin vesicles. Failure to demonstrate proportional release of DBH and CA may suggest several possibilities, without ruling out the concept of quantal release of CA: (1) that small molecules such as CA and ATP are extruded much faster than large protein molecules like DBH and chromogranin in the short span of

time available; (2) that the so-called soluble proteins (including DBH) found in *in vitro* lysates of vesicles are not actually soluble within the vesicles *in vivo*; (3) that the small quantities of DBH released in the perfusate may be a consequence of the normal degradation of vesicles; and finally, (4) secretion of proteins from vesicles storing CA may be species-dependent.

In order to elucidate further the nature of the secretory process, additional studies were made on adrenals of cats pre-treated with reserpine, which markedly depletes the CA stores of this organ. A single injection of reserpine (10 mg/kg) reduced adrenal CA by 75 % in 24 hr, while DBH activity remained within the control level. At 48 hr, when the CA level was reduced to 5 % of the control value, the DBH activity increased by three-fold. Viveros, Arqueros, Connett & Kirshner (1969) found in rabbits that a high dose of reserpine (5 mg/kg) caused a reduction of CA and DBH activity of the gland, and attributed the depletion to neurogenic stimulation by this dose of reserpine. In the cat, 30 % of the total adrenal DBH is readily released in the soluble lysate of chromaffin vesicles, and should have been lost from the gland during neurogenic stimulation by this very high dose of reserpine if soluble proteins were released with CA. On the contrary, the DBH activity remained unchanged initially, and then rose to above the control value when the adrenal CA were maximally depleted on days 2–5 after reserpine. We conclude, on the basis of these results, that CA are lost from the gland following neurogenic stimulation with reserpine, but not soluble proteins.

Stimulation of the cat adrenal gland with ACh (10^{-4} M) resulted in a comparable release of DBH, both from normal and from reserpine-treated cats. Perfusion of glands from cats pre-treated with reserpine 24 hr previously with ACh released only one-third the amount of CA normally released from a control gland, but DBH release was similar to the control output. A measure of the responsiveness of the reserpinized gland to ACh stimulation was the fact that the same amount of DBH activity was released as from a control gland, suggesting thereby that the vesicles of the reserpinized gland were viable. At 2, 3, 4 and 5 days after reserpine, when the endogenous CA levels were markedly depressed while DBH activity increased by as much as 5 times, stimulation with ACh released negligible amounts of CA but increasing amounts of DBH in such a manner that the release of DBH was directly related to the endogenous DBH activity of the gland at all times. Furthermore, release of DBH from these reserpinized glands was also calcium-dependent, as in untreated glands. It is pertinent that when the endogenous CA content has been largely lost due to reserpine action, the adrenal gland still responds normally by releasing DBH in the perfusate. This can be

interpreted to mean that the chromaffin vesicles, although devoid of CA, can still participate in the secretory cycle. These experiments therefore suggest that secretion of CA and DBH is not coupled, and may occur independently of each other. Moreover, assuming that the increase in DBH activity after reserpine was due to the appearance of new vesicles, the fact that the increased endogenous DBH activity was reflected in the release of more DBH activity by ACh suggests that the new vesicles which have not yet acquired any CA also can participate in the secretory cycle.

Subcellular fractionation studies on adrenal glands obtained from normal and reserpine-pre-treated cats showed that even though pre-treatment with reserpine enhanced the DBH activity by 3- to 7-fold, the proportion of soluble to membrane-bound DBH in the 26,000 *g* pellet remained relatively constant at 30 % in the case of normal and reserpine-pre-treated cats. This result suggests that the increased DBH activity is present in the vesicles in soluble and insoluble forms, as in control glands.

Even though the evidence presented so far does not allow for secretion by release of all the soluble components of the vesicle, information obtained from fractionation studies using a discontinuous gradient gives some support to the concept of quantal release (all or none) of CA. Normally 30 % of the total CA was present in fraction *C* and 40 % in fraction *E*, while DBH activity was distributed evenly at 38 % in the two fractions. After severe depletion of adrenal CA by reserpine, DBH activity, which, was unaffected one day after reserpine, was mainly localized in fraction *C*, while only 14 % was in fraction *E*. We suggest that the cause of increase in DBH activity of fraction *C* after reserpine has been due to the presence of intact lighter vesicles which have lost CA probably in an all-or-none manner and cannot move through 1·6 M sucrose. The DBH activity of the gland increases substantially on the 2nd day after reserpine, and most of the increase in DBH activity appears in fraction *C*. This leads to a further increase in the ratio of DBH activity in fraction *C*/*E*. These results also show that new vesicles not having acquired CA, or vesicles depleted of CA, appear in fraction *C*, and this fraction may not necessarily represent broken vesicular membranes.

Electron microscopic and biochemical studies have shown that the storage vesicle membranes are retained within the chromaffin cell following depletion of CA by reserpine (De Robertis & Vaz Ferreira, 1957; Trifaro, Poisner & Douglas, 1967). It is an intriguing question whether empty vesicular membranes left behind during secretion are re-utilized to regain their normal levels of CA. Dixon, Kirpekar & Garcia (1973) suggested, on the basis of studies on CA and DBH recovery in adrenals of rats treated with reserpine or reserpine plus CO_2, that empty vesicular membranes were re-utilized. The present experiments in reserpinized cats further

reinforce this suggestion. We have shown that one day after reserpine treatment, when the endogenous adrenal CA levels were markedly reduced, CA release evoked by ACh was only one-third of the control output. However, at this time endogenous DBH levels were unchanged and the DBH release evoked by ACh was comparable to the release evoked from untreated glands. Moreover, release of DBH from reserpinized glands was also calcium-dependent, as in control glands. This observation suggests that even though reserpine blocked further synthesis and storage of CA in 'old' vesicles, these vesicles responded normally to ACh by secreting DBH. Therefore, the chances are that the same vesicles may be re-used for synthesis and storage of CA after the pharmacological disappearance of reserpine from the gland. By the same line of reasoning, the vesicles involved in a normal physiological secretory process may be re-used several times for storage and synthesis of CA before they are finally destroyed.

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