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Acetylcholine-Induced Stimulation of Catecholamine Recovery in Denervated Rat Adrenals after Reserpine-Induced Depletion

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SUMMARY

The present studies evaluate neurogenic factors affecting the rate of catecholamine recovery following depletion in the rat adrenal medulla. Rats with their left adrenal glands denervated received reserpine, 2.5 mg/kg intraperitoneally, daily for 3 consecutive days. The catecholamine contents of the denervated glands were lowered by 54%, and those of the intact glands, by 80%. Four days after the last injection of reserpine the intact glands had recovered their normal catecholamine content, but the denervated glands were still significantly depleted.

Rats treated with reserpine and with repeated intravenous injections of acetylcholine 1 day after the last reserpine injection completely recovered their catecholamine levels 4 days after the last injection of reserpine, in both intact and denervated glands.

The denervated glands showed no increase in tyrosine hydroxylase activity after reserpine treatment, while the enzyme levels of the intact glands rose to 350% of controls. Treatment with acetylcholine increased the tyrosine hydroxylase activity in denervated glands to 180–265% of controls, and that of intact glands to 550–600% of control values.

Dopamine β-hydroxylase activity was increased by 70% in denervated glands following reserpine treatment, while in the intact glands it increased by 160%. In contrast to its effect on tyrosine hydroxylase activity, acetylcholine had no effect on dopamine β-hydroxylase activity in denervated glands of animals examined 2 days after completion of reserpine treatment, and it caused only a 25% increase in the enzyme activity of animals 4 days after reserpine administration.

The data show that the delay in recovery of catecholamine content observed after reserpine treatment in denervated rat adrenals can be overcome by exposing the gland to its normal secretagogue, acetylcholine.

INTRODUCTION

Two reports suggest that the nerve supply to the adrenal glands affects the rate of recovery of their catecholamine content following depletion with insulin (1) or reserpine (2). Hökfelt (1) found that denervation of the left adrenal glands of rabbits 15 hr after the administration of insulin resulted in a significantly smaller recovery of the catecholamine content when these glands were examined 6 days after the insulin treatment. Kroneberg and Schumann (2) also found that denervating the adrenal gland 3 days after reserpine treatment delayed the recovery of the catecholamine content. These studies indicate a trophic effect of the splanchnic innervation upon the adrenal medulla.

Tyrosine hydroxylase appears to be the

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rate-limiting enzyme in the formation of catecholamines in sympathetically innervated tissues as well as in the adrenal medulla (3, 4). A wide variety of treatments, such as administration of insulin (5–7), reserpine (8), 6-hydroxydopamine (9), or phenoxybenzamine (9), sinoaortic denervation (10), and immobilization stress (11), which increase neurogenic stimulation of the adrenal medulla, also cause a rise in the tyrosine hydroxylase activity of intact adrenal glands but do not increase the activity of the enzyme in denervated glands. It has recently been shown (6) that the administration of acetylcholine itself will cause an increase in the tyrosine hydroxylase activity in denervated glands as well as in intact glands.

The present study was undertaken to determine the effects of prior denervation on catecholamine recovery following reserpine-induced depletion, and to determine the effects on the rate of catecholamine recovery of treatment with acetylcholine, which causes an increase in tyrosine hydroxylase activity in the denervated glands (6).

METHODS

Male Sprague-Dawley rats (200–275 g at time of death) with denervated left adrenals were obtained from Zivic-Miller Laboratories, Allison Park, Pa. Animals had been denervated for 12–21 days when killed. Control denervated animals of the same age were always assayed along with the treated animals. Four groups of animals were used: (a) untreated denervated controls; (b) denervated animals treated with reserpine, 2.5 mg/kg intraperitoneally, for 3 consecutive days; (c) same as (b) but receiving acetylcholine 1 day after the last injection of reserpine; and (d) same as (b) but receiving acetylcholine for 3 consecutive days, starting 1 day after the last injection of reserpine. Acetylcholine was administered as previously reported (6). Five doses each of acetylcholine chloride, 20 mg/kg, together with eserine, 0.1 mg/kg, in 0.15 M sodium chloride were administered via the tail vein at 30-min intervals. Atropine sulfate, 0.5 mg/kg, was given intraperitoneally 15 min before the first and immediately after the third acetylcholine plus eserine injection to protect against the muscarinic effects of acetylcholine. Between injections the animals were kept in separate cages, and were returned briefly to restraining cages for drug treatment.

Preparation of homogenates. The rats were killed between 10 a.m. and noon by a blow on the base of the skull, then bled at the neck. The adrenal glands were removed, cleaned of fat and connective tissue, and homogenized in ice-cold 0.3 M sucrose (one gland in 2.5 ml), using Potter-Elvehjem all-glass homogenizers. Aliquots were removed for total catecholamine and total dopamine β-hydroxylase determinations. The remainder of the homogenate was centrifuged at 26,000 × *g* for 20 min, and the supernatant fraction was assayed for tyrosine hydroxylase activity. Less than 5% of the total hydroxylase activity was found in the resuspended pellets.

Assay of dopamine β-hydroxylase. Dopamine β-hydroxylase was assayed as previously described (6). The reaction mixture contained sodium phosphate buffer, pH 5.8, 200 mM; fumarate, pH 5.8, 120 mM; ascorbate, 2.0 mM; ATP, 0.1 mM; tranylcypromine, 1.0 mM; tritiated tyramine, generally labeled (7.3 Ci/mmole), 0.01 mM; catalase, 600 units; Triton X-100, 0.5%; and 0.4 ml of the sucrose homogenate in a final volume of 1.0 ml. In addition, each reaction mixture contained a final concentration of 4 mM *p*-hydroxymercuribenzoate to inactivate endogenous inhibitors. The mixtures were incubated in air at 37° for 30 min, and the reaction was stopped by addition of 1 ml of 7% perchloric acid. After centrifugation, a 1-ml aliquot of the supernatant fluid was assayed for the amount of octopamine formed by the periodate oxidation method of Friedman and Kaufman (12). Five per cent glycerol was used to stop the periodate oxidation. Under these conditions dopamine β-hydroxylase activity was linear with tissue concentration.

Tyrosine hydroxylase. Tyrosine hydroxylase activity was assayed as described by Nagatsu, Levitt, and Udenfriend (13). The reaction mixture contained sodium acetate buffer, pH 6.0, 200 mM; ferrous ammonium sulfate, 0.5 mM; tranylcypromine, 0.1

mM; 2-amino-6,7-dimethyl-4-hydroxy-4,6,7,8-tetrahydropteridine hydrochloride, 2.0 mM; 2-mercaptoethanol, 20 mM; 3,5-tritiated tyrosine, 0.2 mM (specific activity, 31.6 Ci/mmole); and 0.4 ml of the 26,000 × *g* sucrose supernatant fraction in a final volume of 1 ml. The mixtures were incubated in air at 37° for 15 min. The reaction was stopped by the addition of 0.05 ml of glacial acetic acid.

Catecholamines. Aliquots of the adrenal fractions (0.1 ml) were added to 2 ml of 3.5% perchloric acid. The mixture was centrifuged, and the supernatant fluid was decanted, diluted, and assayed for catecholamine content without further treatment as described previously (14).

Protein determinations. Protein was determined by the method of Lowry *et al.* (15), using bovine serum albumin as a standard.

Statistical methods. The results are expressed on a per gland basis. Student's *t*-test was used to determine statistical significance.

Materials. Tritiated tyrosine and tyramine were obtained from New England Nuclear Corporation. Acetylcholine chloride was obtained from Calbiochem; eserine sulfate (physostigmine), from Mann Research Laboratories; atropine sulfate, from Eli Lilly and Company; and reserpine, from Ciba Pharmaceutical Company.

RESULTS

Effect of reserpine treatment on rat adrenal catecholamine levels. Catecholamine levels were markedly depleted in both denervated and intact glands as a result of reserpine treatment (Fig. 1). The greater depletion observed in intact glands was probably due to increased neural stimulation of the gland, in addition to the direct effect of reserpine in blocking catecholamine uptake into storage vesicles (5).

Four days after the last reserpine injection, the intact gland, even though initially more depleted than the denervated gland, recovered to control levels while the denervated gland still showed a significant depletion and required about 10 days to regain its normal catecholamine content.

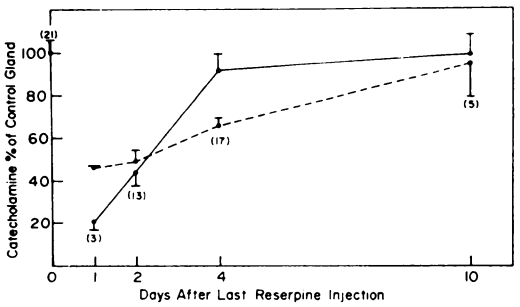

FIG. 1. *Effect of reserpine on rat adrenal catecholamine levels*

Rats with a denervated left adrenal gland were given reserpine, 2.5 mg/kg intraperitoneally, for 3 consecutive days. Numbers in parentheses refer to the number of animals in each group. Dashed lines refer to the denervated glands, and the solid lines to the intact glands. Vertical bars represent standard errors of the means. Control values were: denervated glands, 9.6 ± 0.6 μg/gland; intact glands, 12.8 ± 0.8 μg/gland. The controls at day 0 received no reserpine.

Between days 1 and 2 after the last reserpine injection, the levels of catecholamines in the denervated glands barely increased, from 4.4 to 4.7 μg/gland, a 7% rise. The catecholamine levels of the intact glands rose from 2.6 to 5.5 μg/gland, a 112% increase, during the same time period.

Between days 2 and 4 after the last reserpine injection, the levels of catecholamines in the denervated glands rose from 4.7 to 6.3 μg/gland, a 34% increase. In the intact glands the catecholamine content increased by 110%, from 5.5 to 11.6 μg/gland, during the same time period.

Effect of reserpine treatment on rat adrenal tyrosine hydroxylase activity. Tyrosine hydroxylase activity was increased to 425% of control values in the intact adrenal glands 24 hr after the last reserpine injection, but was unchanged in the denervated glands. The activity remained high in intact glands for 4 days and then declined to 125% of controls 10 days after the last reserpine injection (Fig. 2).

Effect of reserpine treatment on rat adrenal dopamine β-hydroxylase activity. Two days after the last injection of reserpine, dopamine β-hydroxylase activity increased to 260% of the control values in the intact

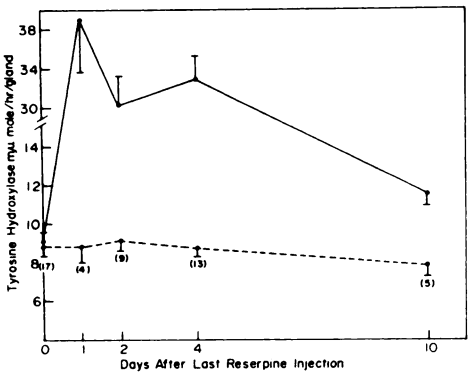

FIG. 2. *Effect of reserpine on rat adrenal tyrosine hydroxylase activity*

Rats with a denervated left adrenal gland were given reserpine, 2.5 mg/kg intraperitoneally, for 3 consecutive days. Numbers in parentheses refer to the number of animals in each group. Vertical bars represent the standard errors of the means. Dashed lines refer to the denervated glands, and the solid lines to the intact glands. The controls at day 0 received no reserpine.

adrenals and to 170% of the control values in the denervated glands. Thereafter, the activities declined to the control levels by approximately 10 days (Fig. 3).

Effect of acetylcholine on adrenal catecholamine levels. Four days after reserpine treatment, the intact adrenal glands recovered their normal catecholamine content whereas the denervated glands regained only 66% of their normal content even though the denervated glands had been depleted to a lesser extent. After treatment with acetylcholine for 1 day after reserpine administration, the catecholamine levels in denervated glands, measured 4 days after the last injection of reserpine, were within normal limits (Table 1).

There was an apparent 24-hr lag in the recovery of catecholamines in both the intact and denervated glands following treatment with acetylcholine, perhaps because of acetylcholine-stimulated secretion of catecholamines. There was no increase in catecholamine content 24 hr after administration of acetylcholine (2 days after reserpine), although replenishment of the catecholamine stores in the denervated glands kept pace with the recovery in the intact glands during the 2–4-day post-reserpine period. After treatment with reserpine only, the denervated glands increased their catecholamine stores in the next 2–4-day period, from 4.7 to 6.3 μg/gland, a rise of 1.6 μg/gland. The denervated glands of reserpine- and acetylcholine-treated animals increased their catecholamine levels by 5.3 μg/gland, from 4.1 to 9.4, during the same period.

Treatment with acetylcholine for 3 consecutive days after reserpine appeared to delay the recovery of catecholamines in both the intact and denervated glands, but this was probably due to secretion of catecholamines as a result of the repeated stimulation.

Effect of acetylcholine on adrenal tyrosine hydroxylase levels. Repeated injections of acetylcholine 1 day after the last reserpine injection increased the tyrosine hydroxylase activity of denervated glands to 180% of the control levels when assayed 24 hr later (Table 1). The activity remained at the higher level for at least 2 days more. After treatment with acetylcholine, the levels of tyrosine hydroxylase in the intact glands rose to levels higher than those due to reserpine treatment alone. Injections of acetylcholine on 3 consecutive days, start-

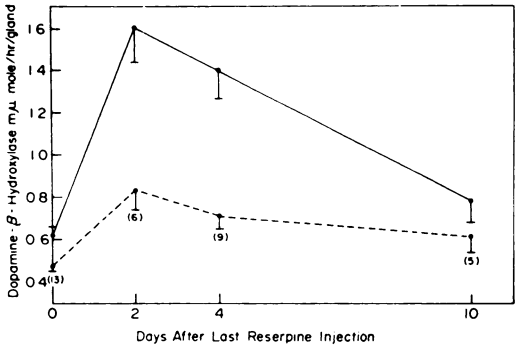

FIG. 3. *Effect of reserpine on rat adrenal dopamine β-hydroxylase activity*

Rats with a denervated left adrenal gland were given reserpine, 2.5 mg/kg intraperitoneally, for 3 consecutive days. Numbers in parentheses refer to the number of animals in each group. Vertical bars represent the standard errors of the means. Dashed lines refer to the denervated glands, and the solid lines to the intact glands. The controls at day 0 received no reserpine.

TABLE 1

Effects of reserpine and acetylcholine on rat adrenals

Animals were treated with reserpine for 3 consecutive days. Twenty-four hours after the last reserpine injection the rats received acetylcholine as described in METHODS, either for 1 day or for 3 consecutive days. The animals in groups A, B, and C were killed 2, 4, and 10 days, respectively, after the last injection of reserpine. The number of animals in each group is shown in parentheses. Values are means ± standard errors.

Group	Treatment	Tyrosine hydroxylase		Dopamine β-hydroxylase		Catecholamines	
		Intact	Denervated	Intact	Denervated	Intact	Denervated
		mμmoles/hr/gland		*mμmole/hr/gland*		*μg/gland*	
	None	9.1 ± 0.5 (17)	8.8 ± 0.5	0.62 ± 0.04 (13)	0.47 ± 0.02	12.2 ± 0.8 (21)	9.6 ± 0.6
		% controls		*% controls*		*% controls*	
A	Reserpine	332 ± 33 (9)	103 ± 6[a]	258 ± 34 (6)	176 ± 19	43 ± 5 (13)	49 ± 5
	Reserpine + acetylcholine, 1 day	549 ± 26 (15)	180 ± 10	290 ± 26 (15)	157 ± 11	39 ± 2 (14)	43 ± 3
B	Reserpine	360 ± 27 (13)	99 ± 4[a]	226 ± 21 (9)	151 ± 13	91 ± 8[a] (13)	66 ± 3
	Reserpine + acetylcholine, 1 day	400 ± 24 (14)	198 ± 16	258 ± 13 (14)	189 ± 13	102 ± 6[a] (14)	98 ± 6[a]
	Reserpine + acetylcholine, 3 days	593 ± 55 (9)	266 ± 17	306 ± 31 (9)	202 ± 21	85 ± 7[a] (9)	70 ± 4
C	Reserpine	126 ± 6 (5)	89 ± 7[a]	126 ± 16[a] (5)	130 ± 15[a]	98 ± 9[a] (5)	94 ± 15[a]
	Reserpine + acetylcholine, 3 days	162 ± 6 (4)	149 ± 17	126 ± 11[a] (4)	140 ± 11	93 ± 10[a] (4)	103 ± 10[a]

[a] Values not significantly different from the corresponding control glands of untreated animals. All other data have p values from <0.05 to <0.001.

ing 1 day after the last reserpine injection, caused even greater changes in the tyrosine hydroxylase activity in both the denervated and intact glands. The activity rose to 266% of the control values in the denervated glands, and to 600% of the control levels in the intact glands, 24 hr after the last injection of acetylcholine.

All glands which had shown increases in tyrosine hydroxylase activity also exhibited a decline toward control levels when assayed 10 days after the last reserpine injection.

Effect of acetylcholine on adrenal dopamine β-hydroxylase levels. In contrast to tyrosine hydroxylase, which showed no increase in denervated glands after reserpine treatment and a substantial increase after acetylcholine administration (Table 1), the dopamine β-hydroxylase activity in denervated glands was increased by reserpine treatment alone and did not rise markedly following acetylcholine treatment. After the reserpine injections, treatment with acetylcholine for 1 day did not cause a further increase in dopamine β-hydroxylase. However, 4 days after the last injection of reserpine, the enzyme activity of the denervated glands of those animals which received acetylcholine for 1 day or on 3 consecutive days was 25–35% higher ($p < 0.05$) than in those animals which received only reserpine. The increased levels of dopamine β-hydroxylase declined toward control levels, as was the case with tyrosine hydroxylase, by 10 days after reserpine treatment.

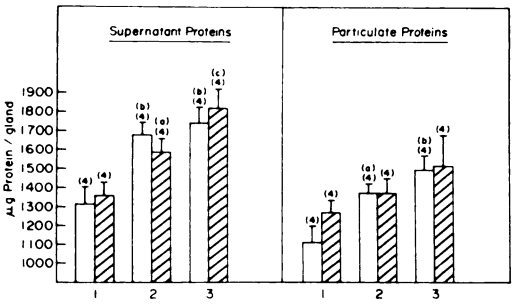

FIG. 4. *Effect of reserpine on protein content of rat adrenals*

Rats with a denervated left adrenal gland were given reserpine, 2.5 mg/kg intraperitoneally, for 3 consecutive days. Numbers in parentheses refer to the number of animals in each group. All animals were killed 2 days after the last injection of reserpine. Numbers on the abscissa refer to the following groups: *1*, no treatment; *2*, reserpine-treated animals; *3*, same as *2*, treated additionally with acetylcholine 1 day following the last injection of reserpine. The unshaded bars refer to the denervated glands, and the hatched bars to the intact glands. Vertical bars represent the standard errors of the means. Particulate proteins are the total protein present in the 26,000 × *g* pellet; supernatant proteins are the total proteins present in the 26,000 × *g* supernatant fraction. Letters in parentheses above the bars refer to the following statistical values compared to corresponding glands of untreated animals: *a*, $p < 0.05$; *b*, $p < 0.02$; *c*, $p < 0.01$.

Effect of reserpine treatment on rat adrenal protein content. The data in Fig. 4 indicate that the protein content of the 26,000 × *g* supernatant fraction was slightly increased over control levels in both intact and denervated glands 2 days after the last reserpine injection. The 26,000 × *g* pellet protein content rose significantly only in the denervated glands, but the total amount was not greater than that of intact glands. In no case did the acetylcholine treatment cause any further change in protein content beyond that caused by the reserpine treatment alone.

DISCUSSION

Adrenal denervation results in a slower rate of recovery of catecholamines to control levels in glands which have been depleted either before (1, 2) or after denervation, as reported in the present studies. The data presented here indicate that exposure to the normal adrenal secretagogue, acetylcholine, is a sufficient stimulus to increase the activity of the cellular mechanisms involved in catecholamine recovery.

Restoration of adrenal catecholamine content requires not only biosynthesis of the amines but also recovery of the ability of the storage vesicles to incorporate and store them. Neurogenic stimulation, whether induced by large doses of reserpine or by insulin administration, causes a discharge of the entire soluble contents of the catecholamine storage vesicle, leaving the vesicle membrane within the cell (16–23).

The soluble contents include, in addition to catecholamines, soluble dopamine β-hydroxylase, chromogranins, and ATP, components which are involved in the synthesis as well as the storage of the amines. After insulin administration, the the dopamine β-hydroxylase activity of the glands returned to normal levels within 48 hr, while recovery of the catecholamine content and the ability of the storage vesicles to incorporate exogenous amines paralleled each other and required 6 days to reach control levels (5). These observations suggest that there is a considerable delay between the re-formation of storage vesicles and their ability to store catecholamines.

The activities of the biosynthetic enzymes may also play a critical role in determining the recovery rate of catecholamines. Tyrosine hydroxylase activity rises markedly following neurogenic stimulation of intact adrenal glands and in denervated glands after administration of acetylcholine. These increases in tyrosine hydroxylase can be blocked by treatment with agents which inhibit protein synthesis, such as actinomycin D and cycloheximide (6, 24), and appear to be genetically controlled events occurring in response to neurogenic stimulation. Twenty-four hours after the last injection of reserpine there is a marked elevation in tyrosine hydroxylase activity in intact adrenals, but complete recovery of catecholamine content requires 3 days longer. This additional period of time may be required for restoration of the ability of the cell to store the catecholamines within the storage vesicles.

The increased rate of catecholamine recovery does not appear to be related to the higher enzymatic activity of dopamine β-hydroxylase, since a marked rise in activity was observed in the denervated adrenals after reserpine treatment alone, and the subsequent administration of acetylcholine, which markedly increased the recovery rate, caused only a modest elevation in dopamine β-hydroxylase activity. It is possible that an even larger increase in dopamine β-hydroxylase activity was not observed because of loss of the enzyme in response to stimulation. The increase in dopamine β-hydroxylase may have been due to synthesis of vesicles *de novo* while the cell retained the enzyme activity associated with the old vesicles (5, 23). The nature of the increase in dopamine β-hydroxylase activity in the denervated glands following reserpine treatment has not been characterized. It may involve protein synthesis, but other possibilities, such as activation of enzyme activity or destruction of an endogenous inhibitor, may be involved. It is of interest that the increases in activity of tyrosine hydroxylase and dopamine β-hydroxylase are not linked.

The observed increases in protein content following treatment with reserpine and with reserpine plus acetylcholine do not reflect a response to neurogenic stimulation, because similar increases were found in both intact and denervated glands. Since the medulla represents only about 20% of the adrenal gland weight (11), the observed changes in protein content may have been due to changes in the cortex as well as the medulla.

In summary, the results indicate that exposure to acetylcholine is sufficient to stimulate catecholamine recovery in depleted glands. The concurrent increase in tyrosine hydroxylase activity as well as repair or synthesis of vesicles *de novo* may well play a role in the increased catecholamine recovery in acetylcholine-treated glands, but whether or not other factors may also contribute to increased catecholamine recovery is still to be determined.

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REFERENCES

1. B. Hökfelt, *Acta Physiol. Scand.* **25**(Suppl.), 92 (1951).
2. G. Kroneberg and H. J. Schumann, *Experientia* **15**, 234 (1959).
3. M. Levitt, S. Spector, A. Sjoerdsma and S. Udenfriend, *J. Pharmacol. Exp. Ther.* **148**, 1 (1965).
4. G. Rosenfeld, L. Leeper and S. Udenfriend, *Arch. Biochem. Biophys.* **74**, 252 (1958).
5. O. H. Viveros, L. Arqueros, R. J. Connett and N. Kirshner, *Mol. Pharmacol.* **5**, 69 (1969).

6. R. L. Patrick and N. Kirshner, *Mol. Pharmacol.* **7**, 87 (1971).
7. N. Weiner and W. F. Mosimann, *Biochem. Pharmacol.* **19**, 1189 (1970).
8. R. A. Mueller, H. Thoenen and J. Axelrod, *J. Pharmacol. Exp. Ther.* **169**, 74 (1969).
9. H. Thoenen, R. A. Mueller and J. Axelrod, *Nature* **221**, 1264 (1969).
10. V. DeQuattro, R. Maronde, T. Nagatsu and N. Alexander, *Fed. Proc.* **27**, 1240 (1968).
11. R. Kvetňansky, V. K. Weise and I. J. Kopin, *Endocrinology* **87**, 744 (1970).
12. S. Friedman and S. Kaufman, *J. Biol. Chem.* **240**, 4763 (1965).
13. T. Nagatsu, M. Levitt and S. Undenfriend, *Anal. Biochem.* **9**, 122 (1964).
14. O. H. Viveros, L. Arqueros, R. J. Connett and N. Kirshner, *Mol. Pharmacol.* **5**, 60 (1969).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. W. W. Douglas, A. M. Poisner and R. P. Rubin, *J. Physiol. (London)* **179**, 130 (1965).
17. W. W. Douglas and A. M. Poisner, *J. Physiol. (London)* **183**, 236 (1966).
18. P. Banks and K. Helle, *Biochem. J.* **97**, 40c (1965).
19. N. Kirshner, H. J. Sage, W. J. Smith and A. G. Kirshner, *Science* **154**, 529 (1966).
20. N. Kirshner, H. J. Sage and W. J. Smith, *Mol. Pharmacol.* **3**, 254 (1967).
21. H. Blaschko, R. Comline, F. Schneider, M. Silver and A. D. Smith, *Nature* **215**, 58 (1967).
22. F. H. Schneider, A. D. Smith and H. Winkler, *Brit. J. Pharmacol. Chemother.* **31**, 94 (1967).
23. N. Kirshner and O. H. Viveros, *in* "New Aspects of Storage and Release Mechanisms of Catecholamines" (H.-J. Schumann and G. Kroneberg, eds.), p. 78. Springer-Verlag New York, 1970.
24. R. A. Mueller, H. Thoenen and J. Axelrod, *Mol. Pharmacol.* **5**, 463 (1969).