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Secretion from the cortex-free bovine adrenal medulla

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1. The isolated bovine adrenal medulla was prepared from whole glands by careful removal of the cortex. The tissue was perfused with physiological salt solution and stimulated with a variety of secretogogues.

2. Catecholamines were secreted from the tissue upon stimulation with carbamylcholine, nicotine sulphate, acetylcholine bromide, histamine dihydrochloride, (+)-amphetamine sulphate and potassium chloride.

3. Carbachol-induced secretion of catecholamines was reduced in the presence of either hexamethonium bromide or tetracaine hydrochloride, during perfusion with calcium-free perfusion fluid, or during perfusion at low temperature.

4. Stimulation of the medulla with carbachol also led to secretion of chromaffin granule protein and acid deoxyribonuclease. The relationships between catecholamines secreted and the amounts of these proteins secreted were similar to the corresponding values for the perfused whole bovine adrenal gland. Perfusate lactate dehydrogenase activity and perfusate haemoglobin content were unchanged after carbachol stimulation.

5. It is concluded that there are no differences in the mechanisms for catecholamine secretion from the cortex-free perfused medulla and the perfused whole gland.

The adrenal gland, perfused either *in situ* or *in vitro*, has been widely used to investigate adrenergic mechanisms. Stimulation of the gland with drugs that are similar in their actions to acetylcholine, or by the splanchnic nerve, causes the secretion of a variety of chromaffin cell constituents. These secretion products include catecholamines (see Coupland, 1965), adenine nucleotides and their metabolites (Douglas, Poisner & Rubin, 1965), chromaffin granule protein (Banks & Helle, 1965; Kirshner, Sage & Smith, 1967; Blaschko, Comline, Schneider, Silver & Smith, 1967; Schneider, Smith & Winkler, 1967; Viveros, Arqueros & Kirshner, 1968; Schneider, 1969) and enzymes associated with lysosomes (Schneider, 1968). In all these studies, the intact adrenal gland has been used, consisting of cortex and medulla. It is conceivable that certain secretion products might derive from cortical tissues, especially in regard to secretion of total protein and lysosomal enzymes. The presence of the cortex might also affect various adrenergic mechanisms of the medulla, as has been shown to be the case with catecholamines

biosynthesis (Wurtman & Axelrod, 1966). For these reasons an attempt was made to perfuse and to stimulate the bovine adrenal medulla which has been freed of surrounding cortical tissue. The results of these experiments are presented in this paper.

Methods

Perfusion

Bovine adrenal glands weighing between 10 and 25 g were obtained approximately 15 min after the animals were killed, and kept in ice for 30–60 min until perfusion was begun. Bovine adrenal glands were perfused and stimulated as described previously (Schneider *et al.*, 1967).

The isolated medulla was prepared from the glands by carefully cutting away the cortex with scissors, taking care not to cut into the medulla. A cannula was inserted into the aperture of the adrenal vein, and tied in place with a ligature around a small amount of aortic and cortical tissue left surrounding the adrenal vein opening. The tissue was perfused in a retrograde fashion with oxygenated (95% oxygen: 5% carbon dioxide) fluid. The perfusion medium consisted of 137 mM NaCl, 2·68 mM KCl, 1·80 mM $CaCl_2$, 0·28 mM NaH_2PO_4, 0·001 mM $MgCl_2$, 11·60 mM $NaHCO_3$ and 5·56 mM glucose. Injection of methylene blue (1 ml. of a 1% aqueous solution) into the perfusion fluid immediately before it entered the tissue showed that the small amount of cortical tissue left was not perfused.

The drugs used to stimulate catecholamine secretion from the tissue were dissolved in 2 ml. of perfusion fluid and infused continuously for a period of 2 min into the perfusion medium with a Harvard infusion pump. The drugs were infused at a point immediately before the fluid entered the tissue. The perfusate flow was maintained at a constant rate for any given preparation and was generally between 10 and 15 ml./min.

Assays

Perfusates (1·5 ml.) were analysed for catecholamines by the colorimetric method of Euler & Hamberg (1949), using citrate phosphate buffer at pH 6·0 (McIlvaine, 1921). In the calculation of the results, catecholamines were expressed as μ-moles of adrenaline. Protein was precipitated by trichloroacetic acid (final concentration of 5% (w/v)), and measured by the microbiuret method (Goa, 1953). Protein assays were standardized with bovine serum albumin.

For the analysis of cholesterol, the perfusates were evaporated to dryness at 40° C under reduced pressure, extracted by the method of Folch, Lees & Sloane Stanley (1957), and assayed by the method of Zlatkis, Zak & Boyle (1953). The optical density at 420 mμ of the perfusates mixed with an equal volume of 0·1 N HCl was used as a measure of the amount of haemoglobin present. Lactate dehydrogenase activity was measured by the method of Wróblewski & La Due (1955). Deoxyribonuclease (DNase) activity of unconcentrated perfusates was measured by the method of Smith & Winkler (1966).

Polyarylamide gel electrophoresis (Clarke, 1964) was performed on perfusates which had been reduced in volume by dialysis against solid sucrose at 4° C. The gels were stained with 1% amido-Schwartz dye in 7·5% acetic acid. After de-staining in 7·5% acetic acid the gels were scanned with a Gilford linear transport system

coupled to a Gilford photometer and a Gilford Model 2000 recorder. Soluble lysates were prepared from bovine adrenal medulla chromaffin granules according to Smith & Winkler (1967).

Drugs

Drugs used were: carbachol (carbamylcholine chloride), acetylcholine bromide, nicotine sulphate, histamine dihydrochloride, (+)-amphetamine sulphate, hexamethonium iodide and tetracaine hydrochloride.

Results

Spontaneous loss of tissue constituents

Figure 1 shows that the amounts of catecholamines, protein, haemoglobin and lactate dehydrogenase activity in perfusates were high immediately after beginning perfusion, then decreased to a fairly stable level after 20–50 min of perfusion. Haemoglobin levels were usually below the limit of detection after perfusion for an hour. The level of perfusate lactate dehydrogenase activity was more variable in this regard, but usually could be detected in perfusates even after 3 or 4 hr of perfusion. Preparations were routinely perfused 1 hr before samples were collected for assay.

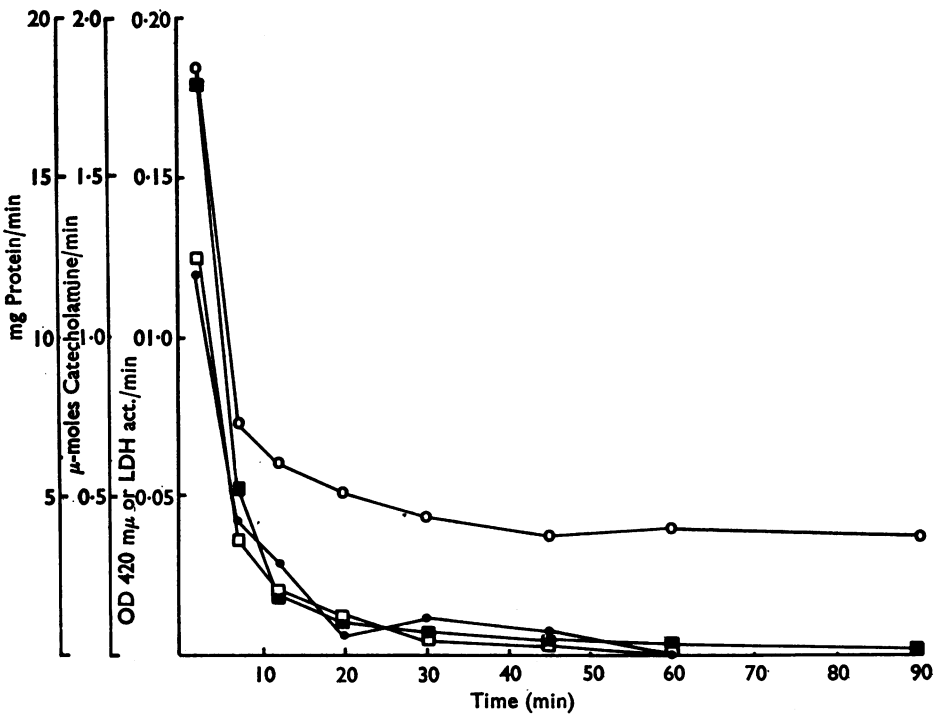

FIG. 1. Catecholamine (○), haemoglobin (420 mμ, ●) lactate dehydrogenase activity (LDH □) and protein (■) in perfusion fluid from an unstimulated isolated bovine adrenal medulla. The flow rate of perfusion was approximately 10 ml./min.

Responses to carbachol

Carbachol stimulation of the medulla caused the secretion of catecholamines. Figure 2 shows the time course for secretion, the dose-response relationship for carbachol, and responses to repeated injections of the drug. The dose-response relationships obtained from several other preparations were similar to that shown in Fig. 2. Stimulation of the medulla with carbachol also induced the release of protein (Table 1). The ratios of the carbachol-induced increase in perfusate catecholamines to the increase in protein are the same for medulla and whole gland. There was no detectable increase in perfusate lactate dehydrogenase activity ($n=8$) or in the amount of haemoglobin ($n=6$) in perfusates after stimulation of the medulla preparation with 2·4 mg of carbachol. The amount of cholesterol in perfusates increased $48{\cdot}1 \pm 25{\cdot}8\%$ ($n=11$) after stimulation with carbachol (2·4 mg). The ratio of the increase in catecholamines (μ-moles) to the increase in cholesterol

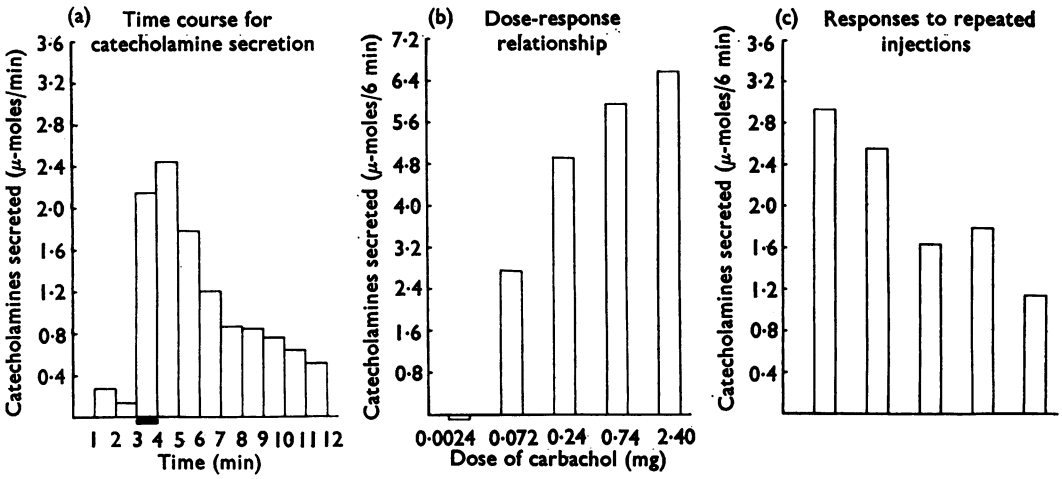

FIG. 2. Carbachol-induced secretion of catecholamines from the isolated bovine adrenal medulla. Panels (a), (b) and (c) represent three separate experiments. In (a), the height of each column represents the amount of catecholamines secreted in 1 min; the horizontal bar represents the period during which 2·4 mg of carbachol was infused. In (b) and (c), the injections consisted of 2·4 mg of carbachol in 2 ml. of perfusion fluid injected over a 2 min period.

TABLE 1. *Catecholamines and protein of perfusates*

Carbachol (mg)	Isolated medulla	Whole gland
1·2	$5{\cdot}62 \pm 0{\cdot}37$ (8)	$4{\cdot}97 \pm 0{\cdot}49$ (5)
2·4	$6{\cdot}22 \pm 0{\cdot}50$ (40)	$5{\cdot}93 \pm 0{\cdot}49$ (7)
3·6	$6{\cdot}17 \pm 0{\cdot}54$ (6)	$5{\cdot}91 \pm 0{\cdot}30$ (52)

$P>0{\cdot}10$ for isolated medulla versus gland for all doses of carbachol.
The values represent the increase in catecholamines (μ-moles) divided by the increase in protein (mg) obtained after stimulation with the doses of carbachol indicated. The figures are the means $\pm$ standard error, and the numbers of experiments are shown in parentheses. Collection periods were 6 min.

(μ-moles) in perfusates was $0{\cdot}35 \pm 0{\cdot}18$ ($n = 11$). The corresponding ratio for bovine adrenal medulla chromaffin granules is 0·0098 (Smith, 1968).

The proteins of the perfusates were examined by polyacrylamide gel electrophoresis (Fig. 3). The densitometer scans of the polyacrylamide gels showed that the primary chromogranin bands for the chromaffin granule soluble lysate were present in the perfusate collected during the stimulation period and were not detectable in the perfusate collected during the control period. Gel electrophoresis also

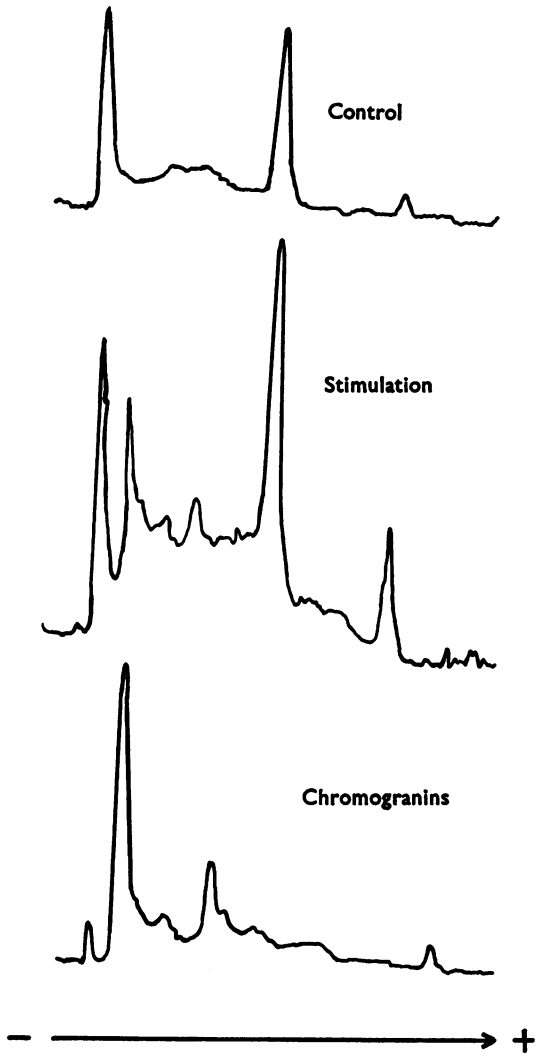

FIG. 3. Polyacrylamide gel electrophoresis of proteins from perfusates of the bovine adrenal gland and of chromogranins from the soluble lysate of chromaffin granules. After staining the gels with amido-Schwartz dye the intensities of the bands were measured by transmitted light with a densitometer. The arrow indicates the direction in which the proteins migrated. Perfusates were concentrated to one-tenth their original volume by dialysis against solid sucrose at 4° C.

showed that there were proteins other than chromogranins present in perfusates collected during control and stimulation periods.

Inhibition of carbachol-induced catecholamine secretion

Carbachol-induced secretion of catecholamines was reduced in the presence of hexamethonium, or tetracaine (Fig. 4). Secretion in response to carbachol stimulation was also prevented when the tissue was perfused with calcium-free perfusion fluid (Fig. 4). Exposure of the medulla to calcium ($CaCl_2$, 32·2 mg in 2 min) during perfusion with calcium-free fluid increased the catecholamines in the perfusate by 61% ($n=2$).

A reduction of the temperature of the perfusion fluid also caused a decrease in the amount of catecholamines secreted after carbachol stimulation (Fig. 4).

Catecholamine secretion induced by other secretogogues

A large number of drugs stimulate catecholamine secretion from the adrenal gland (see Coupland, 1965; Garrett, Osswald, Rodrigues-Pereira & Guimaraes, 1965; Rubin & Jaanus, 1966), and a few of these were tested for their ability to stimulate catecholamine release from the perfused isolated medulla. Shown in Fig. 5 is an experiment in which the preparation was stimulated with carbachol, nicotine, (+)-amphetamine, potassium chloride, histamine and acetylcholine. Each of these drugs stimulated catecholamine secretion from the medulla.

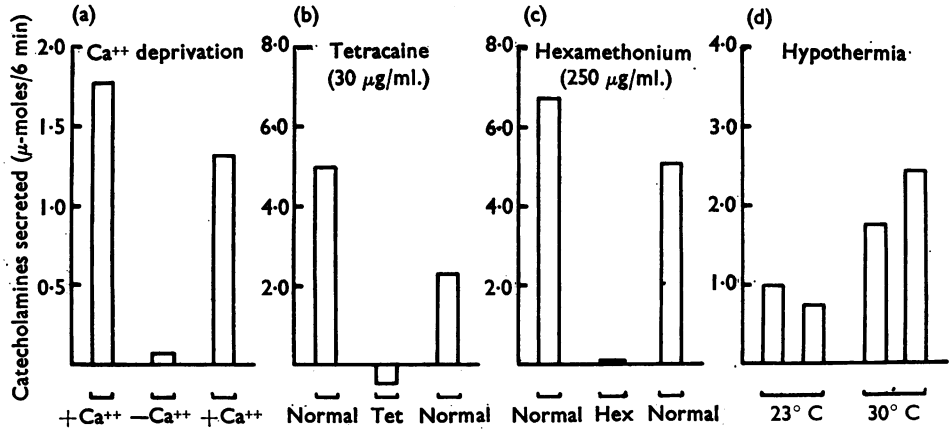

FIG. 4. Inhibition of carbachol-induced catecholamine secretion from the isolated bovine adrenal medulla. Carbachol was injected in a dose of 2·4 mg. The height of each column represents the increase in catecholamines above control levels; collection periods were 6 min. Panels (a), (b), (c) and (d) represent separate experiments. In (a), (b) and (c) the tissues were perfused for 30 min with calcium-free fluid, tetracaine-containing fluid or hexamethonium-containing fluid, respectively, before further stimulation. The tissues were perfused with normal perfusion fluid 30 min before stimulation for the third time. In (d), 30 min was allowed before stimulation after changing the temperature of the perfusion fluid.

Carbachol-induced secretion of deoxyribonuclease activity

Stimulation of the isolated bovine adrenal gland with carbachol or with acetylcholine causes the secretion of enzymes typical of those found in lysosomes (Schneider, 1968). Accordingly, perfusates from the isolated medulla and from the perfused whole gland were assayed for activity of the lysosomal enzyme acid deoxyribonuclease (Table 2). The relationship between catecholamines and deoxyribonuclease activity for perfusates from the isolated medulla is not different from the corresponding values for the whole gland.

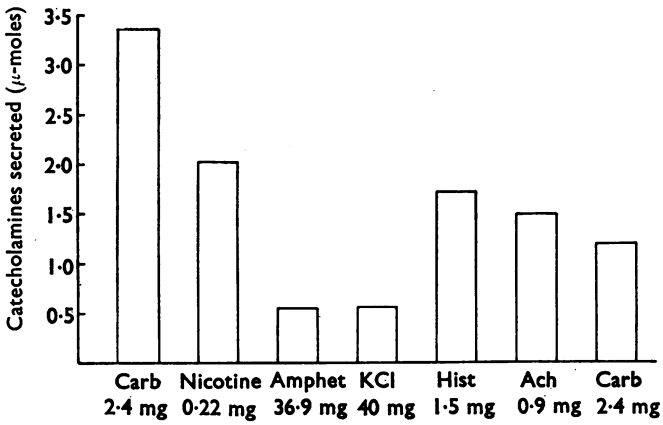

FIG. 5. Catecholamine secretion from the isolated bovine adrenal medulla. The height of each column represents the increase in catecholamines above control levels; collection periods were 6 min. Drugs were injected in 2 ml. of perfusion fluid over a period of 2 min. Carb, Carbachol; Amphet, (+)-amphetamine sulphate; Hist, histamine dihydrochloride; ACh, acetylcholine bromide.

TABLE 2. *Ratios of catecholamines to deoxyribonuclease activity in perfusates after carbachol stimulation*

Carbachol (mg)	Isolated medulla	Whole gland
1·2	850·1±142·5 (7)	991·0±256·6 (4)
2·4	918·6±124·5 (12)	853·5±193·4 (7)
3·6	897·2±187·5 (6)	917·7±111·1 (28)

$P>0·05$ for isolated medulla versus whole gland for all doses of carbachol.
The values represent the increase in catecholamines (μ-moles) divided by the increase in deoxyribonuclease activity (μ-moles/min) obtained after stimulation with the doses of carbachol indicated. The figures are the means±standard errors, and the numbers of experiments are shown in parentheses. Collection periods were 6 min.

Discussion

The observations presented in this paper show that secretion of catecholamines from the isolated bovine adrenal medulla occurs by exocytosis. Secretion of this type is consistent with the findings that catecholamines and protein are secreted in a ratio similar to the corresponding ratio of lysates from isolated bovine adrenal medulla chromaffin granules (Schneider *et al.*, 1967), that chromogranins, the soluble proteins of chromaffin granules, are present in perfusates obtained upon stimulation, and that little cholesterol, a constituent of the chromaffin granule membrane, is secreted with the catecholamines. Exocytosis has been shown to be the mechanism by which catecholamines are secreted from the perfused whole bovine adrenal gland (Schneider *et al.*, 1967).

The marked similarity between the biochemical features of catecholamine secretion from the isolated adrenal medulla and from the whole adrenal gland indicates that the presence of the cortex is of little importance for the process of exocytosis. These results also serve to establish further that the medulla is the source of the major portion of the protein secreted from the whole gland.

The similarities in the secretion of catecholamines from these two preparations extend also to the pharmacological features of the secretion process. Secretogogues that release catecholamines from the perfused whole gland also cause secretion from the iolated medulla, and agents or conditions that reduce carbachol-induced catecholamine secretion from the whole gland act similarly on the isolated medulla. The requirement for calcium is the same in both preparations.

Carbachol stimulation of the perfused whole gland also causes secretion of lysosomal enzymes (Schneider, 1967). The availability of the perfused isolated medulla made it possible to assess how much, if any, of the secreted acid hydrolases came from the cortex. It appears that essentially all the acid deoxyribonuclease secreted from the whole gland derives from the medulla, because the relationship between catecholamines and deoxyribonuclease secreted on stimulation with carbachol is the same for both preparations.

It is possible to conclude from these studies that previous observations on catecholamine and lysosomal enzyme secretion from the perfused isolated whole gland do in fact represent events occurring in the medulla, and that these events are not influenced by the presence of the cortex.

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REFERENCES

BANKS, P. & HELLE, K. (1965). The release of protein from the stimulated adrenal medulla. *Biochem. J.*, **97**, 40–41*c*.

BLASCHKO, H., COMLINE, R. S., SCHNEIDER, F. H., SILVER, M. & SMITH, A. D. (1967). Secretion of chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Nature, Lond.*, **215**, 58–59.

CLARKE, J. T. (1964). Simplified " disc " (polyacrylamide gel) electrophoresis. *Ann. N.Y. Acad. Sci.*, **121**, 428–436.

COUPLAND, R. E. (1965). *The Natural History of the Chromaffin Cell.* London: Longmans Green.

DOUGLAS, W. W., POISNER, A. M. & RUBIN, R. P. (1965). Efflux of adenine nucleotides from perfused adrenal glands exposed to nicotine and other chromaffin cell stimulants. *J. Physiol., Lond.*, **179**, 130–137.

EULER, U. S. VON & HAMBERG, U. (1949). Colorimetric determination of noradrenaline and adrenaline. *Acta physiol. scand.*, **19**, 74–84.

FOLCH, J., LEES, M. & SLOANE STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.*, **226**, 497–509.

GARRETT, J., OSSWALD, W., RODRIGUES-PEREIRA, E. & GUIMARAES, S. (1965). Catecholamine release from the isolated perfused adrenal gland. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **250**, 325–336.

GOA, J. (1953). A microbiuret method for protein determination; determination of total protein in cerebrospinal fluid. *Scand. J. clin. Lab. Invest.*, **5**, 218–222.

KIRSHNER, N., SAGE, H. J. & SMITH, W. J. (1967). Mechanism of secretion from the adrenal medulla II. Release of catecholamines and storage vesicle protein in response to chemical stimulation. *Mol. Pharmac.*, **3**, 254–265.

MCILVAINE, T. C. (1921). A buffer system for colorimetric comparison. *J. biol. Chem.*, **49**, 183–186.

RUBIN, R. P. & JAANUS, S. D. (1966). A study of the release of catecholamines from the adrenal medulla by indirectly acting sympathomimetic amines. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **254**, 125–137.

SCHNEIDER, F. H. (1968). Observations on the release of lysosomal enzymes from the isolated bovine adrenal gland. *Biochem. Pharmac.*, **17**, 848–851.

SCHNEIDER, F. H. (1969). Drug-induced release of catecholamines, soluble protein and chromagranin A from the isolated bovine adrenal gland. *Biochem. Pharmac.*, **18**, 101–107.

SCHNEIDER, F. H., SMITH, A. D. & WINKLER, H. (1967). Secretion from the adrenal medulla: Biochemical evidence for exocytosis. *Br. J. Pharmac. Chemother.*, **31**, 94–104.

SMITH, A. D. (1968). Biochemistry of adrenal chromaffin granules. In *The Interaction of Drugs and Subcellular Components in Animal Cells*, ed. Campbell, P. N. Boston: Little, Brown and Company.

SMITH, A. D. & WINKLER, H. (1966). The localization of lysosomal enzymes in chromaffin tissue *J. Physiol., Lond.*, **183**, 179–188.

SMITH, A. D. & WINKLER, H. (1967). Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem. J.*, **103**, 483–492.

WRÓBLEWSKI, F. & LA DUE, J. S. (1955). Lactate dehydrogenase activity in blood. *Proc. Soc. exp. Biol. Med.*, **90**, 210–213.

WURTMAN, R. J. & AXELROD, J. (1966). Control of enzymatic synthesis of adrenaline in the adrenal medulla by adrenal cortical steroids. *J. biol. Chem.*, **241**, 2301–2305.

VIVEROS, O. H., ARQUEROS, L. & KIRSHNER, N. (1968). Release of catecholamines and dopamine β-oxidase from the adrenal medulla. *Life Sci. Oxford*, **7**, 609–618.

ZLATKIS, A., ZAK, B. & BOYLE, A. J. (1953). A new method for the direct determination of serum cholesterol. *J. lab. clin. Med.*, **41**, 486–492.

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