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SECRETION FROM THE ADRENAL MEDULLA: BIOCHEMICAL EVIDENCE FOR EXOCYTOSIS

BY

F. H. SCHNEIDER,* A. D. SMITH AND H. WINKLER †

From the Department of Pharmacology, Oxford University

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Catecholamines are secreted from the adrenal gland under many physiological and experimental conditions (for a review see Coupland, 1965a). In order to explain catecholamine secretion at a cellular level, De Robertis & Vaz Ferreira (1957) suggested that the amines of the adrenal medulla may be released from the intracellular storage granules directly into the extracellular spaces by a process which was called reverse pinocytosis. This suggestion was based on evidence obtained from electron microscopy, which has since been confirmed by Coupland (1965b). Since that time several workers have presented biochemical evidence in agreement with this hypothesis. Douglas and his co-workers (see Douglas, 1966) have shown that stimulation of the perfused cat adrenal by acetylcholine leads to the secretion of adenine nucleotides and their metabolites; the molar ratio of catecholamines to adenine derivatives in the perfusates was similar to that in chromaffin granules. Similar observations have been made using the perfused bovine adrenal gland (Banks, 1966). Furthermore, Banks & Helle (1965), using an immunochemical method, have shown that the major protein component of the chromaffin granules is also secreted upon stimulation with carbachol. This finding has been confirmed with quantitative immunochemical methods (Kirshner, Sage, Smith & Kirshner, 1966; Sage, Smith & Kirshner, 1967). The same protein was also secreted from the calf adrenal gland *in situ* upon splanchnic nerve stimulation (Blaschko, Comline, Schneider, Silver & Smith, 1967); the soluble proteins of chromaffin granules were called chromogranins by these authors. Accordingly, we shall call the major component of these proteins "chromogranin A".

Additional biochemical evidence in support of the idea that catecholamine release occurs by reverse pinocytosis is presented in this paper. For this type of secretion mechanism the term exocytosis has been proposed by de Duve (1963).

METHODS

Perfused adrenal gland

Bovine adrenal glands, weighing between 8 and 15.5 g, were obtained approximately 20 min after the animals were killed, and kept in ice for about 30 min until perfusion was begun. The

* Present address: Department of Pharmacology, University of Colorado Medical Center, Denver, Colorado, U.S.A.

† Permanent address: Pharmakologisches Institut der Universität, Innsbruck, Austria.

glands were perfused in a retrograde manner through the adrenal vein (Hechter, Jacobsen, Schenker, Levy, Jeanloz, Marshall & Pincus, 1953; Banks, 1965) with Tyrode solution (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) at 37°, and gassed with a mixture of 95% O_2 and 5% CO_2. Flow rates varied between 7 and 20 ml./min, with a rate of 10 to 12 ml./min in most experiments. Secretion of catecholamines was induced by injection of 15 mM-carbamoylcholine chloride (Carbachol, British Drug Houses Ltd.) into the perfusion fluid immediately before the fluid entered the gland. Carbachol was injected in volumes of 0.4 ml. or less, in a series of six separate injections, each separated by 30 sec.

Chemical assays

Perfusates from the glands and extracts of chromaffin granules (prepared according to Smith & Winkler, 1967a, b) were analysed for catecholamines by the colorimetric method of Euler & Hamberg (1949), using citrate-phosphate buffer (McIlvaine, 1921). In the calculation of the results, catecholamines were expressed in terms of adrenaline. Protein was precipitated by trichloroacetic acid (final concentration of 5% (W/V)), and measured by the microbiuret method (Goa, 1953).

For the analysis of lipids, the perfusates were evaporated to dryness at 37° under reduced pressure and extracted by the method of Folch, Lees & Sloane-Stanley (1957). Fatty acids were determined by the method of Sheath (1965), and cholesterol by the method of Zlatkis, Zak & Boyle (1953). Separation by thin-layer chromatography and subsequent analysis of phospholipids was carried out according to the method described by Skipski, Peterson & Barclay (1964). Lipid phosphorus was determined directly on silica scraped from the plates by the method of Bartlett (1959).

Starch gel electrophoresis (Poulik, 1957) and amino acid analysis (following hydrolysis of the protein for 17 hr at 110° in 6N HCl) were performed on perfusates and soluble lysates which had been reduced in volume by ultrafiltration (Sober, Gutter, Wyckoff & Peterson, 1956) at 4° C and dialysed for a minimum of 12 hr against tris Na-succinate buffer (I 0.015, pH 5.9).

Immunological procedures

Rabbit antiserum was prepared against the major soluble protein (chromogranin A) of chromaffin granules, which was purified by the method of Smith & Winkler (1967b). The animals were injected (intramuscularly) with 0.5 ml. pure protein (1.6 mg/ml.) mixed with an equal volume of Freund's complete adjuvant. Injections were repeated 7 days later with half as much material. On the 34th day after the initial injection the animals were given subcutaneous injections of a mixture of 0.5 ml. protein solution (1.6 mg/ml.) and 25 mg aluminium phosphate. Blood was collected from the marginal ear vein between 3 and 4 weeks after the final injection. Thereafter subcutaneous injections of the protein-aluminium phosphate mixture was given every 4 to 5 weeks and the rabbits were bled 2 weeks after each injection. The antiserum was stored at 4° in the presence of 0.01% merthiolate and 25% undiluted complement, and was used at dilutions of $^1/_{50}$–$^1/_{100}$; it was incubated for 30 min at 56° immediately before use in order to destroy complement.

A microcomplement fixation method similar to that described by Fulton & Dumbell (1949) was used to titrate the amount of antigen. The diluent was made by the alternative procedure of Kabat & Mayer (1961), and contained 0.1% gelatine. Complement fixation was carried out in Perspex agglutination trays (Prestware Ltd., London), each well of which contained complement (0.1 ml.) antigen (0.1 ml.) and antibody (0.1 ml.) or the appropriate control. Complement was used at a dilution which provided 2½ $C'H_{50}$. The trays were incubated for 1½ hr at 37°. One-tenth of a ml. of 2% (V/V) sheep red blood cells, sensitized with an optimum amount of haemolysin for 10 min at 37° immediately before use, was then added, and incubation continued at 37° for an additional 1½ hr. Dilutions of antigen and antiserum were used which were free of anticomplement activity. The titre was obtained from the dilution of the antigen at which approximately 50% of the cells were haemolyzed. The amount of antigenic protein was determined by comparing the titres of the unknown with that of the standard antigen solutions. Since doubling dilutions were used, this method was accurate within the limits of 0.5 to 2 times the observed value.

Lactate dehydrogenase activity

This was determined by the method of, and expressed in the units of, Wróblewski & La Due (1955).

Materials

Complement (preserved guinea-pig serum), sheep red blood cells (sheep blood in alsever solution), and haemolysin (rabbit haemolytic serum) were purchased from Burroughs Wellcome & Co., London. Freund's adjuvant, complete, was purchased from Difco Laboratories, Detroit.

RESULTS

Catecholamine secretion

Resting secretion of catecholamines from the adrenal gland was low, usually less than 0.1 μ-mole/min. Injection of 15 mM-carbachol (2.4 ml. over a 2½-min period) caused the secretion of large amounts of catecholamines (Fig. 1). The amount of catecholamine

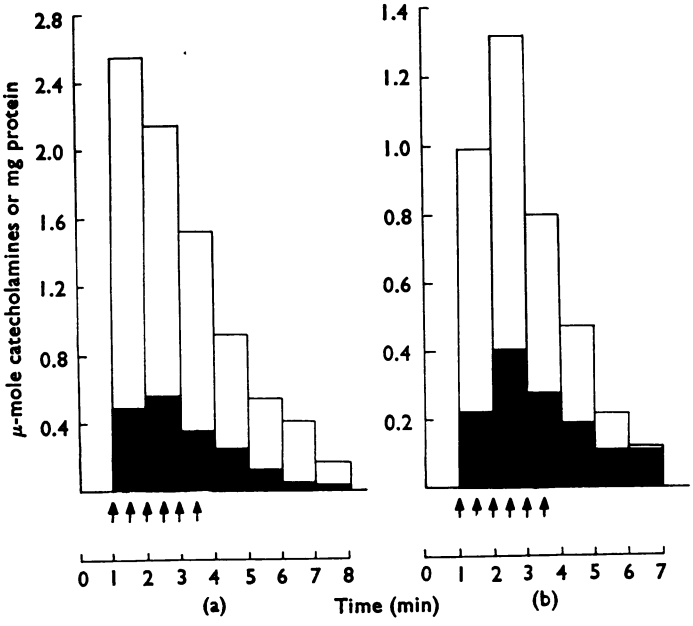

Fig. 1. Secretion of catecholamines and protein from a bovine adrenal gland during two periods of stimulation. The heights of the open columns indicate the increment of the catecholamines and those of the black columns indicate the increment of protein above control level. The arrows indicate the injection of carbamylcholine (see Methods). Experiments (a) and (b) were separated by 2 hr. The flow rate of perfusion fluid was 13 ml./min for experiment (a), and was 9 ml./min for experiment (b). Spontaneous release of catecholamines and of protein, respectively: (a) 0.02 μ-mole/min and 0.43 mg/min, (b) 0.02 μ-mole/min and 0.16 mg/min.

released at different flow rates was similar, although the appearance of the amines in the perfusate was slightly delayed at the lower flow rates. The amount of catecholamine secreted in each stimulation period decreased upon repeated stimulation. The flow rate decreased by up to 15% as a result of the injection of carbachol in 13 out of 14 experiments.

Secretion of protein

The perfusates contained considerable amounts of protein during the first 30 min of perfusion. The amount of protein decreased exponentially, and reached a fairly constant level within 40 to 60 min (Fig. 2). Due to the high protein content of the perfusate

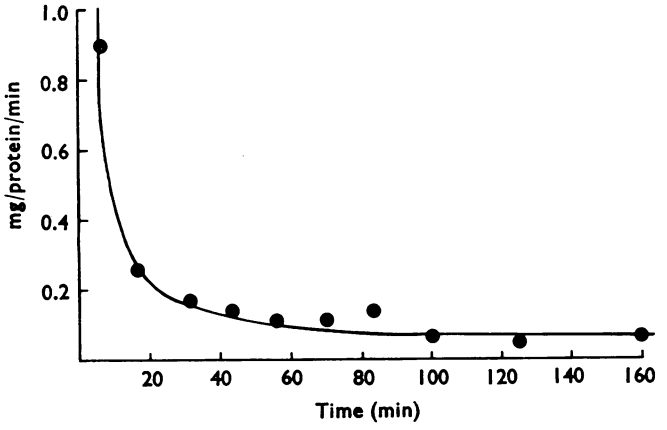

Fig. 2. Protein content of perfusion fluid from a bovine adrenal gland that was not stimulated. Protein was determined by the microbiuret method. The flow rate of perfusion fluid was approximately 14 ml./min.

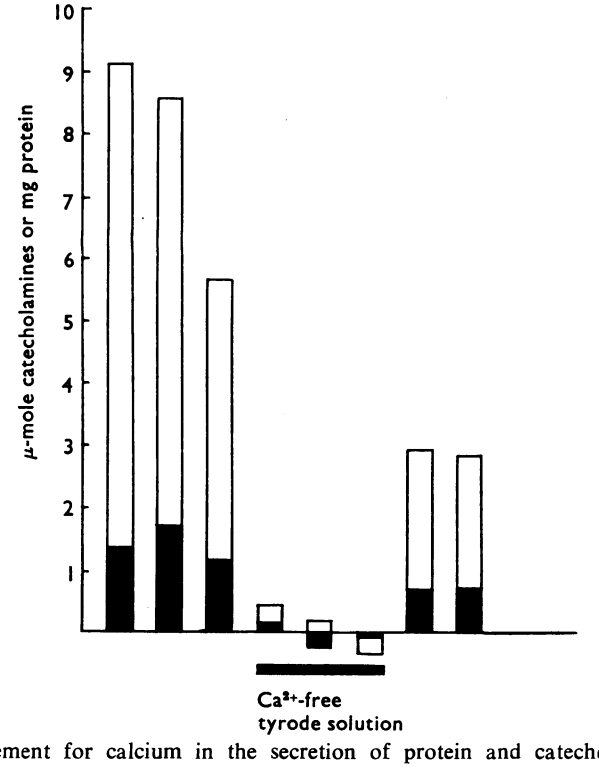

Fig. 3. The requirement for calcium in the secretion of protein and catecholamines induced by carbamylcholine. Each column represents a 3-min stimulation period ; 20 min elapsed between each stimulation period. The heights of the open columns indicate the increment of the catecholamines and those of the black columns indicate the increment of protein above control level. Ca^{2+}-free perfusion fluid was used during (and for 20 min beforehand) the 4th, 5th and 6th periods of stimulation. Normal Tyrode solution was perfused for 20 min before the two final stimulation periods. Between the stimulation periods the amounts of catecholamines and protein, respectively, in the perfusate ranged from 0.2–0.3 μ-mole/min and from 0.1–0.5 mg/min.

during the early stages of perfusion, perfusates were not collected for analysis during the first hour. Stimulation of the gland with carbachol, in addition to inducing catecholamine secretion, caused an increase in the amount of protein in the perfusate, as shown in Fig. 1. The release of protein was almost simultaneous with that of catecholamines.

The dependence of several secretory processes upon the presence of calcium (see Douglas, 1966) prompted an examination of the effects of calcium depletion upon carbachol-induced secretion of protein. Figure 3 shows that protein secretion, as well as catecholamine release, was abolished when calcium was left out of the perfusion medium. When the gland was again perfused with complete Tyrode solution secretion of both protein and hormones could be induced.

Relationship of catecholamine to protein in perfusates

The ratio of the amount of catecholamines to that of protein secreted above the resting level was fairly constant. This ratio depended somewhat upon the length of time for which the perfusate was collected after the initial injection of carbachol, and was close to the ratio of catecholamines to total protein in the soluble lysate of chromaffin granules (see Table 1).

Secretion of specific protein

The complement fixation method enabled us to detect 10 ng of chromogranin A. Analysis of 4 different perfusates showed that about half of the protein secreted in response to carbachol consisted of the specific protein (Table 1). This value is similar

TABLE 1

CATECHOLAMINES AND PROTEINS IN PERFUSATES FROM THE STIMULATED GLAND AND IN SOLUBLE LYSATES OF CHROMAFFIN GRANULES

The amounts of catecholamines and protein secreted during stimulation were obtained by subtracting the amounts of each constituent in the control period from the amounts in the stimulation period perfusates. The figures represent the means (± S.D.) of n determinations. The term chromogranin A refers to the major component of the soluble proteins from chromaffin granules

	μ-mole catecholamines / mg total soluble protein	μ-mole catecholamines / mg chromogranin A	mg chromogranin A × 100 / mg total protein
Soluble lysate of granules	4·8 ±0·6 (n=5)	10·1±3·9 (n=10)	47·6±17·8 (n=10)
Perfusate upon stimulation			
3 min collection	5·4±1·0 (n=13)	11·9±5·0 (n=4)	48·2±20·4 (n=4)
6 min collection	4·8±1·5 (n=19)	—	—

to that obtained upon analysis of the soluble lysate of chromaffin granules. In only one experiment was chromogranin A detected in the perfusate collected during control periods; in this instance it was found to be 3% of the total protein. The ratios of catecholamines (μ-mole) to chromogranin A (mg) in the stimulation period perfusate and in the soluble lysate were almost the same. Similar values for these ratios were obtained by Kirshner *et al.* (1966) and by Sage *et al.* (1967).

Starch gel electrophoresis

Only one major protein component could be detected in the control perfusate by gel electrophoresis and the electrophoretic mobility of this component was the same as that of bovine serum albumin. Analysis of the perfusate collected upon stimulation showed the presence of several proteins, the electrophoretic mobilities of which were identical to corresponding proteins from the chromaffin granules. In addition there was a band

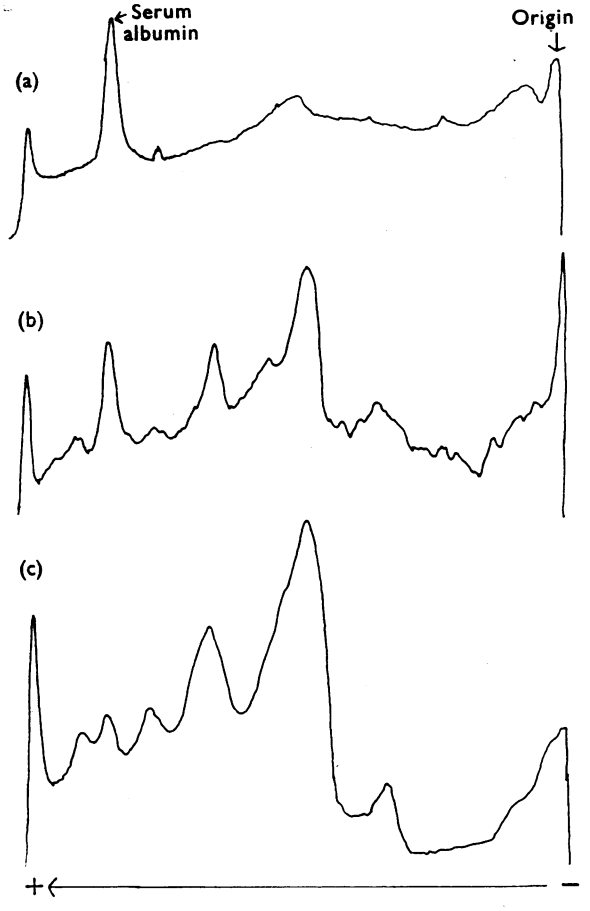

Fig. 4. Starch gel electrophoresis of proteins from perfusates of the bovine adrenal gland and of chromogranins from the soluble lysate of chromaffin granules. After staining the gel with nigrosine (0.05%) the intensities of the bands were measured by transmitted light with a densitometer. The proteins migrated from right to left (a) control period perfusate, (b) stimulation period perfusate, (c) soluble lysate of chromaffin granules.

with the same mobility as serum albumin. Densitometer scans of the starch gels, shown in Fig. 4, illustrate the close similarity between the proteins in the perfusate collected during stimulation and those of the soluble lysate. The major protein band represented 41% of the area under the peaks for the soluble lysate in the scan in Fig. 4 and 43%

of the area in the scan of the stimulation period perfusate gel. Corresponding figures for the next most conspicuous protein band were 23% and 19%.

Amino acid analyses

Amino acid analyses of the proteins from stimulation period perfusates showed some similarity between the composition of these proteins and that of the chromogranins (Table 2). The amino acid composition of the protein in the control perfusate was similar to that of serum proteins. The amino acid composition of the proteins secreted in response to carbachol was calculated by subtracting the amount of each amino acid present in the control perfusate from that of each amino acid in the stimulation period perfusate. The calculated amino acid composition had the same characteristics as that of the soluble proteins from chromaffin granules (Table 2).

TABLE 2

AMINO ACID ANALYSIS OF PROTEINS IN PERFUSATES AND IN SOLUBLE LYSATE OF CHROMAFFIN GRANULES

The values in each column represent g amino acid/100 g protein. The values in the column headed "increment" were calculated by subtracting the absolute amount of each amino acid/unit volume in the control from the corresponding value of the stimulation period perfusate, and dividing the difference by the increment in total protein/unit volume between the two perfusates. The control period perfusate contained 2 mg. protein and the stimulation period perfusate contained 4·43 mg protein

Amino acid	Perfusate (control)	Perfusate (stimulated)	Increment between perfusates	Chromogranins of the soluble lysate
Glu	14·2	22·5	29·3	26·6
Arg	6·2	8·0	9·5	10·7
Lys	10·0	10·2	10·4	8·6
Pro	6·1	8·3	10·1	8·0
Asp	9·8	8·5	7·4	7·7
Leu	9·0	7·0	5·4	6·8
Ser	5·6	5·7	5·8	5·2
Ala	4·7	4·3	4·0	4·2
Gly	3·3	3·8	4·2	3·8
His	4·0	2·5	1·3	3·6
Val	5·4	3·3	1·6	2·7
Thr	5·2	3·0	1·2	2·3
Phe	5·1	3·0	1·3	2·3
Tyr	3·8	3·1	2·5	2·2
Met	1·9	2·0	2·1	1·8
NH_3	1·6	2·9	3·8	1·6
Ile	2·3	1·4	0·7	1·0
Cys	1·8	0·6	—	0·6

Other constituents of the perfusates

The perfusates contained small amounts of phospholipids, fatty acids and cholesterol. Slight and variable increases in the amounts of these constituents were observed upon stimulation. If the increment for the various lipids (μ-mole) was divided by the amount of catecholamines (μ-mole) secreted, then ratios of 1.2 for phospholipid, 0.9 for cholesterol and 1.2 for fatty acids were found. The corresponding ratios in chromaffin granules were, respectively, 211, 114 and 6. The perfusates from stimulation periods contain, therefore, very small amounts of these constituents relative to the amount of catecholamines. In order to determine whether lysolecithin, a characteristic constituent of chromaffin granules

(Blaschko, Firemark, Smith & Winkler, 1967 ; Winkler, Strieder & Ziegler, 1967), was released during stimulation, quantitative thin-layer chromatography of the phospholipids extracted from the perfusates was performed. No significant secretion of lysolecithin could be detected during stimulation in 3 experiments, even though these perfusates each contained about 20 μ-mole of catecholamines. The recovery of lysolecithin (740 μg) injected into the perfusion fluid before it reached the gland was 40%.

There was no increase in the amount of lactate dehydrogenase in the perfusate during stimulation with carbachol (Table 3). It can be calculated from the data in Table 3

TABLE 3

LACTATE DEHYDROGENASE ACTIVITY IN BOVINE ADRENAL MEDULLA AND IN PERFUSATES FROM THE BOVINE ADRENAL GLAND

The high speed supernatant from an homogenate of bovine adrenal medulla was obtained by removing the cell particles by centrifugation at 66×10^5 g-min. Before analysis, the perfusates were concentrated by ultrafiltration at 4°

	Total units of enzyme activity	mg protein	Units of enzyme/mg protein
High speed supernatant (1 ml.)	8,000	4·56	1,754
Control period perfusate (415 ml.)	1,600	8·65	185
Stimulation period perfusate (415 ml.)	1,360	15·83	86

that if only 1% of the amount of protein secreted upon stimulation with carbachol had been derived from the cytoplasmic sap it would have been possible to detect this by means of the lactate dehydrogenase assay.

DISCUSSION

The isolated bovine adrenal gland was used in this work firstly because it has been shown that this preparation will secrete catecholamines (Philippu & Schümann, 1962 ; Banks, 1965), and protein (Banks & Helle, 1965 ; Kirshner *et al.*, 1966 ; Sage *et al.*, 1967) upon stimulation by acetylcholine and carbachol, and secondly because much is known about the biochemistry of the adrenal chromaffin granules of this species. This preparation is therefore well-suited for the study of the question whether catecholamine release is accompanied by the release of other chromaffin granule constituents. Studies have already been made on secretion of adenine nucleotides (Banks, 1966) and of a specific chromaffin granule protein (Banks & Helle, 1965 ; Kirshner *et al.*, 1966 ; Sage *et al.*, 1967) from the bovine adrenal gland.

For further characterization of the protein secreted from the gland it was necessary to find conditions in which the amount of protein in the perfusate prior to stimulation was as low as possible. This was achieved by perfusing the gland for 40 to 60 min before stimulation. The protein occurring in the control perfusate was mainly derived from serum, as shown by starch gel electrophoresis. An increase in the amount of both catecholamine and protein in the perfusate occurred as a result of stimulation with carbachol. The ratios in the perfusates of catecholamine (μ-mole) to the increment of protein (mg) were similar to the corresponding ratios in the soluble lysates of chromaffin

granules. Further evidence of a relationship between the release of catecholamine and that of protein was that neither was secreted when calcium was omitted from the perfusion fluid. The dependence of catecholamine secretion on the presence of calcium has already been demonstrated (Douglas & Rubin, 1961 ; Banks, 1965).

The nature of the protein secreted has been studied by immunochemical and biochemical methods. Using a micro-complement fixation technique the presence of the major component (chromogranin A) of the soluble proteins of chromaffin granules was demonstrated in the perfusate. The antigenic protein comprised about half of the protein secreted above the resting level, which is the same as the amount of this protein found by the immunochemical method in lysates of chromaffin granules. By means of Sephadex chromatography (Smith & Winkler, 1967b) and quantitative starch gel electrophoresis (Winkler, Ziegler & Strieder, 1966) it was found that chromogranin A comprised 38 and 49% respectively of the chromogranins. It is, therefore, unlikely that there is a high degree of cross reaction between the antibody to chromogranin A and the other chromogranins.

Analysis, by starch gel electrophoresis, of the perfusates collected during stimulation demonstrated that several proteins were secreted during stimulation. The electrophoretic mobilities of these components are identical to those of the eight soluble proteins of chromaffin granules. As shown by densitometer scans, these eight proteins in the perfusate were present in the same relative proportions as they were in the soluble lysate. Further confirmation of the identity of the proteins in the perfusates with the chromogranins was obtained by the determination of their amino acid composition. The amino acid analysis of the perfusates collected during stimulation revealed a high content of glutamic acid and proline, but a low content of $\frac{1}{2}$ cysteine ; these are characteristic features of the amino acid composition of the chromogranins.

Since Stjärne (1964), Douglas, Poisner & Rubin (1965) and Banks (1966) have already shown that ATP and its metabolites are secreted along with the catecholamines, we conclude that stimulation of the adrenal medulla leads to secretion of all the soluble constituents of the chromaffin granules.

How is the secretion of the soluble constituents of chromaffin granules brought about ? The discharge of the whole chromaffin granule across the plasma membrane is excluded by our observation that only minute amounts of phospholipids and cholesterol, which are major components of the lipids of chromaffin granules, are present in the perfusate. Furthermore, lysolecithin, a phospholipid characteristic of the chromaffin granule was not secreted. A second possibility is that the contents of the chromaffin granule are discharged into the cytoplasm and then diffuse across the plasma membrane ; this is also unlikely because it would involve a marked increase in the permeability of the plasma membrane to proteins of large molecular size (effective hydrodynamic radius of major component$\simeq$62 Å), and would thus allow cytoplasmic proteins to be secreted. However, the starch gel electrophoresis analyses and the lactate dehydrogenase (effective hydrodynamic radius$\simeq$37 Å) estimations showed that cytoplasmic proteins were not secreted. In addition, Kirshner *et al.* (1966) have reported that the cytoplasmic enzyme phenylethanolamine-N-methyl transferase was not secreted on stimulation.

The remaining possibility is that the secretion of chromaffin granule constituents occurs by a process of exocytosis as was first suggested by de Robertis & Vaz Ferreira (1957).

A similar secretion mechanism was proposed by Palade (1959) for the zymogen granules of the exocrine pancreas. As described by the morphologist, the process of exocytosis involves the fusion of the membrane of the secretory granule with that of the cell. This is the only mechanism that would allow the secretion of both low and high molecular weight components specifically from the chromaffin granules of the adrenal medulla.

SUMMARY

1. The composition of perfusates from isolated bovine adrenal glands has been examined and compared with that of bovine adrenal chromaffin granules. Perfusates were collected during control periods and during periods of stimulation of the gland by carbamylcholine.

2. Stimulation of the glands increased the amount of catecholamines and protein in the perfusates; the secretion of both catecholamines and protein was abolished when Ca^{2+} was omitted from the perfusion fluid.

3. The main protein component (chromogranin A) of chromaffin granules was demonstrated in the stimulation period perfusates by an immunochemical method. This protein, along with the other seven soluble proteins of chromaffin granules, was also detected in these perfusates by means of starch gel electrophoresis. The amino acid composition of the proteins secreted upon stimulation was very similar to that of the soluble proteins (chromogranins) from chromaffin granules.

4. Only small increases in the amounts of phospholipids, cholesterol and fatty acids in the perfusates occurred as a result of stimulation; no increases were observed in the amount of lysolecithin or lactate dehydrogenase.

5. The ratios of the amount of catecholamines both to the total amount of secreted protein and to the amount of chromogranin A in the perfusates were close to the ratios, respectively, of catecholamines to chromogranins and of catecholamines to chromogranin A in isolated chromaffin granules.

6. These results, together with earlier findings, demonstrate that the entire soluble contents of chromaffin granules are secreted from the adrenal medulla upon stimulation. Therefore it is concluded that secretion from the adrenal medulla occurs by means of exocytosis.

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