Membranes of the Adrenal Medulla

BEHAVIOUR OF INSOLUBLE PROTEINS OF CHROMAFFIN GRANULES ON GEL ELECTROPHORESIS

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Washed membranes of bovine adrenal chromaffin granules contained most of the cholesterol and phospholipids of the particle and 22% of the total protein. The protein/lipid ratio was about 0.45 (w/w). Dopamine(3,4-dihydroxyphenethylamine)β-hydroxylase, Mg^{2+}-activated nucleoside triphosphatase and cytochrome b-559 activities were present in the membrane. ATP was the best substrate for the nucleoside triphosphatase, whose pH optimum was 6.4, K_m 7×10^{-4}M and V_{max} 1.8 μmol/h per mg of protein. Treatment of the membranes with various detergents caused a preferential solubilization of protein compared with lipids. Membranes dissolved in sodium dodecyl sulphate or phenol–acetic acid–urea were subjected to polyacrylamide-gel electrophoresis at alkaline and acid pH respectively. The electrophoretic patterns given by the proteins of the chromaffin granule membrane were distinct from those given by the chromogranins, and from those given by mitochondrial and microsomal membrane proteins.

Proteins present in the adrenal chromaffin granule can be divided into two groups: (i) the proteins that are released into the supernatant, together with the hormones, when the particles are lysed by hypo-osmotic shock; (ii) the proteins that are recovered, together with the lipids of the granule membrane, in the insoluble residue after osmotic shock.

In the first detailed study of chromaffin granules it was pointed out that the proportion of soluble protein is higher than usual for a cell particle (Hillarp & Nilson, 1954), and since then much work has been done on the soluble proteins, which have been given the name 'chromogranins' (Blaschko, Comline, Schneider, Silver & Smith, 1967a). Relatively little is known about the insoluble proteins of the chromaffin granule, except that they include cytochrome b-559 (Ichikawa & Yamano, 1965; Banks, 1965), a Mg^{2+}-activated adenosine triphosphatase (Banks, 1965; Kirshner, Kirshner & Kamin, 1966), and dopamine(3,4-dihydroxyphenethylamine)β - hydroxylase (Oka, Kajikawa, Ohuchi, Yoshida & Imaizumi, 1967). The last-named enzyme is approximately equally distributed between the soluble lysate and the insoluble residue after osmotic shock (Duch, Viveros & Kirshner, 1968; Belpaire & Laduron, 1968).

Characterization of the soluble proteins of chromaffin granules led to the discovery that the chromogranins, including the soluble dopamine β-hydroxylase (Viveros, Arqueros & Kirshner, 1968), are quantitatively secreted from the adrenal gland together with the hormones (for reviews see Douglas, 1968; Smith, 1968a). The secretion of the chromogranins is not accompanied by the release of the lipids of the chromaffin granule membrane (Schneider, Smith & Winkler, 1967), which remain behind in the cell (Malamed, Poisner, Trifaró & Douglas, 1968) together with the insoluble dopamine β-hydroxylase (Viveros, Arqueros & Kirshner, 1969). These findings are consistent with the secretion of the chromaffin granule contents by exocytosis, a process that has been observed by electron microscopy (see Diner, 1967).

The final stage of exocytosis involves the fusion of the membrane of the chromaffin granule with the plasma membrane. What is the molecular mechanism of this membrane fusion and what determines its specificity? Before these questions can be answered it is necessary to know more about the chemical composition of the two interacting membranes. The composition of the plasma membrane is not known since it has not been possible to isolate fragments of this membrane from chromaffin cells pure enough for analysis. However, highly purified chromaffin granules can be readily isolated from homogenates of adrenal

medulla, and the lipids of the chromaffin granule membrane have been shown to be remarkable for their high content of lysolecithin (1-acylglycero-phosphorylcholine) (Blaschko, Firemark, Smith & Winkler, 1967b; Winkler, Strieder & Ziegler, 1967; Winkler & Smith, 1968). The present paper describes the preparation and composition of membranes of the chromaffin granule, attempts to isolate a lipoprotein subunit enriched in lysolecithin and gel-electrophoresis experiments that define specific protein components of the chromaffin granule membrane. A preliminary report of some of these results has been published (Winkler & Hörtnagl, 1969).

METHODS

Preparation of subcellular fractions and of chromaffin granule membranes. A large-granule fraction was obtained from homogenates of bovine adrenal medulla as described by Smith & Winkler (1967). To decrease contamination with microsomes (Winkler, 1969), the large-granule fraction was resuspended in 0.3 M-sucrose and the suspension was recentrifuged at 11000g for 20 min. The sediment was resuspended in 0.3 M-sucrose (1 ml contained material from 1-2 g of original tissue) and was fractionated exactly as described by Smith & Winkler (1967). Fraction 2 of these authors was taken as the mitochondrial fraction and fraction 5 (the pink pellet) as chromaffin granules. A microsomal fraction was obtained by centrifuging the combined supernatants, remaining after preparation of the large granules, at 160000g for 60 min.

Each pellet of chromaffin granules (corresponding to 3-4 g of original tissue) was suspended in 2 ml of tris-sodium succinate buffer, pH 5.9 and I 0.015, to lyse the particles. The suspension was frozen and thawed, and then centrifuged at 166000g for 45 min. The supernatant (first soluble lysate) was decanted and the sediment was resuspended, frozen at −15°C for 1 h, thawed and centrifuged again as above. This procedure was repeated a total of five times. The final sediment is called 'washed membranes' of chromaffin granules. The mitochondrial and microsomal fractions were treated in the same way, to prepare washed membranes.

Analytical methods. To determine the protein content of the washed membranes it was necessary to modify the micro-biuret method of Goa (1953) since the membranes did not readily dissolve in NaOH. The sample was first precipitated with trichloroacetic acid (5%, w/v) and the precipitate was dissolved in 3 ml of a solution containing NaOH (3%, w/v) and sodium deoxycholate (2%, w/v). The latter solution was prepared by slowly mixing an equal volume of sodium deoxycholate (4%, w/v) and NaOH (6%, w/v). The biuret reaction was carried out on the protein solution by adding 0.15 ml of Benedict's reagent and the extinction was read at 330 nm after 15 min. Bovine serum albumin dissolved in NaOH (3%, w/v)–sodium deoxycholate (2%, w/v) was used as a standard.

Lipids were extracted by the method of Folch, Lees & Sloane-Stanley (1957); lipid P was determined by Bartlett's (1959) method and total cholesterol by the method of Zlatkis, Zak & Boyle (1953). For the analysis of individual phospholipids, the chloroform extract was subjected to t.l.c. (Skipski, Peterson & Barclay, 1964) and the phosphate content of the spots was determined in the presence of the silica by the method of Bartlett (1959). The catecholamines were measured by the method of von Euler & Hamberg (1949) but with citrate-phosphate buffers (McIlvaine, 1921) instead of acetate buffers.

Glucose 6-phosphatase activity was measured by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) and succinate–tetrazolium reductase by the method of Pennington (1961) as modified by Porteous & Clark (1965). Mg^{2+}-dependent adenosine triphosphatase activity was measured by determination of the P_i formed at 37°C in a reaction mixture containing enzyme (up to 0.2 ml), ATP trisodium salt (5 mM), $MgCl_2$ (5 mM) and tris–sodium succinate buffer, pH 6.4 (50 mM), in a final volume of 1 ml.

Chromogranin A was determined by microcomplement fixation by using rabbit antiserum as described by Schneider et al. (1967).

Dopamine β-hydroxylase activity was assayed by following the formation of [³H]octopamine from [³H]-tyramine (5 μM) in the presence of 500 units of catalase to protect the enzyme from inactivation and in the presence of tranylcypromine sulphate (0.5 mM) to inhibit mono-amine oxidase (Friedman & Kaufman, 1965; Viveros et al. 1968).

For determination of cytochrome content, the membranes (3 mg of protein) were dissolved in 3 ml of sodium deoxycholate (2%, w/v) in glycylglycine–NaOH buffer, pH 7.4 (0.1 M), and the extinction at 425 nm was determined after reduction by excess of sodium dithionite (see Banks, 1965).

Gel electrophoresis. For electrophoresis at acid pH the membrane pellets were dissolved in 0.5 ml of phenol-acetic acid (2:1, w/v) containing urea (final concn. 3 M) and left at room temperature for 20 h before electrophoresis. Gels (5.5%, w/v) were prepared by the method of Takayama, MacLennan, Tzagoloff & Stoner (1966), and were equilibrated (Cotman & Mahler, 1967) against the phenol–acetic acid–water (2:1:1, w/v/v)–urea (5 M) solution for 3 days. Electrophoresis was carried out with a current of 4 mA/tube for 95 min. The upper and the lower electrode compartments contained acetic acid (10%, v/v). The gels were stained in Naphthalene Black 10B (1%, w/v) in acetic acid (7%, v/v) for 1 h and destained overnight in acetic acid (7%, v/v).

For electrophoresis at alkaline pH each membrane pellet was suspended in 0.5 ml of sodium dodecyl sulphate (1%, w/v) and left for 2 h at 2°C. Polyacrylamide gels (3.5%) were prepared with sodium borate–NaOH buffer, pH 9.6 and I 0.036, by the method of Clarke (1964). The same buffer was also used for the electrode vessels, but sodium dodecyl sulphate was added to the upper reservoir to give a final concentration of 1%. Before applying the samples, the gels were pre-run with a current of 2 mA/ tube for 15 min. After application of the sample, a current of 0.75 mA/tube was applied for 10 min, followed by 2 mA/tube for 40 min and then finally 4 mA/tube for 20 min. The gels were stained by the method of De Vito & Santomé (1966).

Treatment of membranes with detergents and enzymes. The various detergents were dissolved in water. Each membrane pellet (2 mg of protein) was suspended in 2 ml of detergent solution; the suspension was left for 2 h in ice and then centrifuged for 45 min at 160 000*g*. The sediment containing the undissolved portion of the membrane was analysed for lipid P and protein. The amount solubilized by the detergent was calculated by comparison with a control sample that had been treated with water. The same procedure was used to study the effect of detergents on protein-free lipid extracts (Folch *et al.* 1957), which were evaporated to dryness and then suspended in detergent solution with a glass rod.

For incubation with trypsin, the membrane pellet (2 mg of protein) was suspended in 2 ml of sodium–potassium phosphate buffer, pH 7.0 (0.1 M), containing trypsin (0.05 mg/ml) and incubated for 10 min at 37°C. Samples were also incubated with pronase (1 mg/ml) in sodium borate–HCl buffer, pH 8.1 and *I* approx. 0.025, at 39°C for 10 min. A control sample was incubated in buffer, and after incubation the suspensions were chilled and then centrifuged at 160 000*g* for 45 min.

Amino acid analysis. Membrane pellets (4 mg of protein) were suspended in 4 ml of constant-boiling HCl. The suspension was placed in a cooling mixture (acetone and solid CO_2) and then dissolved air was removed by a vacuum pump before the tube was sealed. Hydrolysis was carried out for 17 h at 110°C. The amino acids in the hydrolysate were analysed with the Beckman model 120 C amino acid analyser.

Materials. The following substances were used: pronase (B grade; Calbiochem, Los Angeles, Calif., U.S.A.), trypsin (from bovine pancreas, type III; Sigma Chemical Co., St Louis, Mo., U.S.A.), Triton WR-1339 (Serva, Heidelberg, Germany), *N*-cetylpyridinium chloride and sodium deoxycholate (E. Merck A.-G., Darmstadt, Germany), sodium dodecyl sulphate (BDH Chemicals Ltd., Poole, Dorset, U.K.), Naphthalene Black 10B (G. T. Gurr Ltd., London, N.W.9, U.K.), nucleoside triphosphates and catalase [Boehringer Corp. (London) Ltd., London, W.5, U.K.] and [^{3}H]tyramine (*p*-hydroxyphenethylamine) (New England Nuclear Corp., Boston, Mass., U.S.A.).

RESULTS

Composition of washed membranes of chromaffin granules. The washed chromaffin granule membranes were analysed for components of other membranes and were found to be relatively pure. They contained only small amounts of glucose 6-phosphatase activity (0.16 μmol/h per mg of protein) and succinate–tetrazolium reductase activity (1.1 μmol/h per mg of protein). The membranes from the microsomal fraction contained 1.67 units of glucose 6-phosphatase/mg of protein, and those from the mitochondrial fraction contained 7.6 units of succinate–tetrazolium reductase/mg of protein.

To determine the composition of the insoluble residue remaining after osmotic lysis of the chromaffin granules it was first necessary to establish
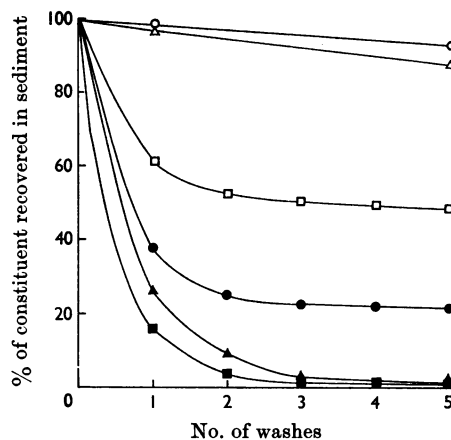

Fig. 1. Effect of repeated washing on distribution of chromaffin granule constituents between the soluble lysate and insoluble residue. Chromaffin granules were suspended in hypo-osmotic buffer, frozen, thawed and centrifuged, five times in all, as described in the Methods section. The ordinate gives the percentage of the amount of each constituent in the original suspension that was recovered in the five sediments. ○, Cytochrome *b*-559; △, phospholipids and cholesterol; □, dopamine β-hydroxylase activity; ●, total protein; ▲, chromogranin A; ■, catecholamines.

how many washes were required to remove the soluble components. Several constituents of the chromaffin granules were analysed in the sediments and supernatants obtained by the osmotic lysis procedure (see the Methods section); the results of these analyses are shown in Fig. 1. The constituents can be divided into three groups: (i) those that are almost quantitatively recovered in the supernatant (catecholamines and chromogranin A); (ii) those that are recovered mainly in the sediment (lipids and cytochrome *b*-559); (iii) those constituents that are only partly soluble (total protein and dopamine β-hydroxylase). Three washes are sufficient to remove all but the last traces of the soluble constituents from the insoluble residue.

The composition of the washed insoluble residue relative to total protein content is given in Table 1. The activity of the enzyme dopamine β-hydroxylase is given as the initial velocity at a substrate concentration of 5 μM [as used by Viveros *et al.* (1968) and Duch *et al.* (1968)]. The $V_{max.}$, determined from a Lineweaver–Burk plot, was 30 μmol/h per mg of protein. The apparent K_m for the enzyme was 1.7×10^{-4} M.

The Mg^{2+}-activated adenosine triphosphatase activity was measured under different experimental conditions, and the optimum pH was found to be 6.4 (Fig. 2). At this pH the $V_{max.}$ was 1.8 μmol of

Table 1. *Composition of washed membranes of chromaffin granules*

The membranes were washed five times and the constituents were analysed by the procedures described in the Methods section.

Protein	1 mg
Lipid P	$2.4 \mu mol$
Cholesterol	$1.66 \mu mol$
Mg^{2+}-stimulated adenosine triphosphatase	$1.8 \mu mol/h$
Dopamine β-hydroxylase (substrate $5 \mu M$)	$0.012 \mu mol/h$
Cytochrome b-559	$0.46 (E_{425})$
Chromogranin A	$0.04 mg$

Fig. 3. Effect of sodium dodecyl sulphate on the solubility of protein and phospholipid of the chromaffin granule membrane. Washed membranes of chromaffin granules (2 mg of protein) were suspended in 2 ml of the detergent solution and, after 2 h in ice, the suspension was centrifuged for 45 min at $160000g$. The sediment was analysed for protein and phospholipid, and the proportion dissolved was obtained by comparison with membranes suspended in water. ○, Protein; ▲, phospholipid.
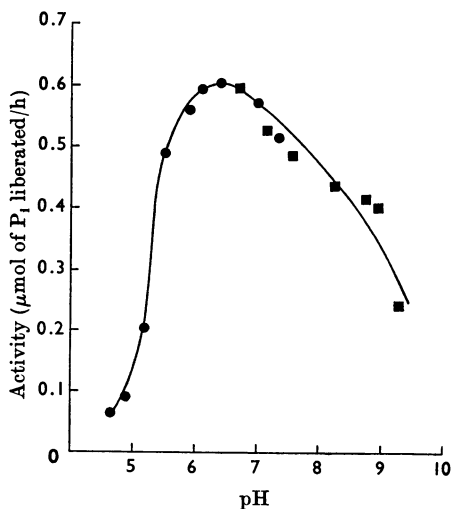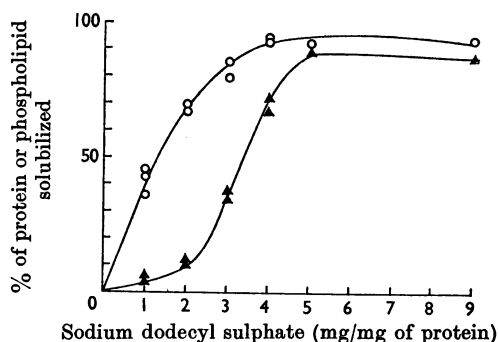

Fig. 2. Effect of pH on activity of Mg^{2+}-stimulated adenosine triphosphatase of chromaffin granule membranes. Washed membranes of chromaffin granules (0.33 mg of protein) were incubated in the medium described in the Methods section, but with buffers of different pH. ●, 0.05 M-Tris-succinate buffer titrated with NaOH; ■, tris–HCl buffer at constant I (0.05).

ATP hydrolysed/h per mg of protein and the apparent K_m was 7×10^{-4} M. The optimum concentration of magnesium chloride was 5 mM and concentrations above 10 mM inhibited the activity. Magnesium chloride at 5 mM caused a sevenfold activation of the adenosine triphosphatase, whereas the same concentration of calcium chloride raised the activity by only 31%. The latter observations confirm those of Banks (1965), who measured the enzymic activity at pH 7.4. The substrate specificity of the enzyme was studied by using buffer at pH 6.4 containing magnesium chloride (5 mM) and

a final substrate concentration of 5 mM. All the substrates tested were hydrolysed less rapidly than ATP; the rates of hydrolysis, relative to that of ATP (100), are as follows: GTP (52), ITP (46), UTP (22), CTP (20), ADP (6) and AMP (2).

Effect of detergents on chromaffin granule membrane lipids and proteins. Experiments were carried out with a variety of detergents to see whether it was possible preferentially to dissolve a lipoprotein enriched in one of the phospholipids. The effect of each detergent on the solubility of total protein and total phospholipid was studied first of all, and the results of a typical experiment with sodium dodecyl sulphate are shown in Fig. 3. At low concentrations of detergent there was preferential solubilization of protein compared with phospholipid, and the same phenomenon was observed with the other detergents (see Table 2). The action of the detergents in dissociating the protein from the phospholipid made it unlikely that individual lipoprotein complexes could be isolated in this way, and this conclusion was supported by analysis of the phospholipid composition of the solubilized phospholipids compared with that of the residual insoluble phospholipids. With each of the detergents there was a preferential solubilization of lysolecithin and this was accompanied (with sodium deoxycholate and sodium dodecyl sulphate) by a retention of sphingomyelin in the insoluble residue. However, a similar pattern of solubilization was obtained when protein-free lipid extracts were treated with low concentrations of detergents and so it cannot be explained by the presence of distinct lipoproteins in the membrane.

Table 2. *Solubilization of chromaffin granule phospholipids and insoluble proteins by detergents*

The first column gives the range of detergent concentrations used: no further solubilization of proteins or phospholipids was obtained with higher concentrations of detergent. The suspensions contained 1 mg of protein/ml.

	Detergent (mg/ mg of protein)	Protein solubilized (%)	Phospholipid solubilized (%)
Sodium dodecyl sulphate	1–5	41–93	5–89
Sodium deoxycholate	1–9	26–70	5–70
Triton WR-1339	5–100	14–41	6–22
N-Cetylpyridinium chloride	0.5–5	18–82	2–82

Gel electrophoresis of insoluble proteins of different subcellular fractions. Since it has not so far been possible to isolate individual lipoprotein subunits of the chromaffin granule membrane an alternative approach to the identification of specific membrane proteins was tried. Analytical gel electrophoresis of the proteins was carried out after dissolution of the membranes in solvents that dissociate lipids from proteins.

Takayama *et al.* (1966) showed that the membranes of mitochondria could be dissolved in a solvent containing phenol, acetic acid and urea. This solvent also dissolved the insoluble residues remaining after lysis of chromaffin granules, mitochondria or microsomes from the adrenal medulla. The dissolved membranes remained in solution if kept at room temperature, but storage at 3°C for more than 4h led to the formation of a sediment composed of needle-shaped crystals. These crystals were isolated from an extract of chromaffin granule membranes, and did not contain any detectable protein. The crystals dissolved in chloroform, and t.l.c. (Freeman & West, 1966) showed that they were composed of cholesterol.

Polyacrylamide-gel electrophoresis (acid pH) of membranes dissolved in phenol–acetic acid–urea was carried out and the results are shown in Plate 1. For comparison, the soluble proteins of chromaffin granules (chromogranins) were also subjected to electrophoresis under the same conditions. The patterns given by the insoluble proteins of chromaffin granules, of mitochondria and of microsomes were readily distinguished from each other and from that given by the chromogranins. It is noteworthy that the pattern of bands given by the chromogranins under acidic conditions, when the proteins migrate as cations, is very similar to that given by the same proteins on gel electrophoresis under alkaline conditions (Strieder, Ziegler, Winkler & Smith, 1968), when the proteins migrate as anions.

The most conspicuous band in the electrophoretogram of the chromogranins is that given by chromogranin A, and a protein with the same electrophoretic mobility is also present in the insoluble residue of chromaffin granules. However, the pattern given by the insoluble proteins differs from that given by the chromogranins both in the number and in the intensity of the slow-moving bands. Only three bands migrate more slowly than chromogranin A in the soluble lysate, whereas nine such bands can be identified in the insoluble residue of chromaffin granules. One of the latter bands is very conspicuous and appears to be the major component of the insoluble proteins. Two other bands given by the insoluble residue are also quite intensely stained, but these remained close to the origin. The electrophoretograms of the insoluble residues of mitochondria and microsomes also contained slow-running components, but they did not contain a conspicuous band with the same mobility as that of the main component of the insoluble proteins of chromaffin granules. It is possible that some of the minor components in the electrophoretograms of each fraction are present owing to contamination with membranes from the other fractions.

Polyacrylamide-gel electrophoresis was also carried out on membranes that had been dissolved by sodium dodecyl sulphate at alkaline pH. The resulting patterns (see Plate 1b) were less complex than the electrophoretograms obtained at acid pH. Thus electrophoresis of the chromaffin granule membranes in the presence of sodium dodecyl sulphate gave ten bands, compared with the 15 bands obtained in the phenol–acetic acid procedure. Also, in contrast with the patterns obtained after electrophoresis under acid conditions, most of the bands migrated at alkaline pH more rapidly than did chromogranin A. However, a conspicuous slow-moving band was present in the insoluble residue, but not in the soluble lysate, of chromaffin granules. It has not yet been possible to identify this slow-running component with one of the three conspicuous slow-running bands in acid gels. Conspicuous slow-running bands were not present in the membranes of the mitochondrial or microsomal fraction after electrophoresis in alkaline sodium dodecyl sulphate. The pattern given by the microsomal fraction was blurred and has not been reproduced.

Effect of proteolytic enzymes on constituents of the chromaffin granule membrane. The presence, in the insoluble residue of chromaffin granules, of a protein with the same electrophoretic mobility as chromogranin A raised the question whether this might be a constituent of the membrane as suggested by Helle & Serck-Hanssen (1969). However, immunochemical analysis of the insoluble residue (see Table 1) showed that only 4% of the insoluble protein cross-reacted with antiserum to chromogranin A. The immunochemical method may have underestimated the amount of chromogranin A since some of the protein might have been inaccessible to the antibody. This possibility was made unlikely by studies on the effect of proteolytic enzymes on the gel-electrophoresis patterns and amino acid composition of the insoluble proteins.

Chromogranin A is rapidly hydrolysed by trypsin (Smith & Kirshner, 1967) and, in agreement with this, it was found that incubation of the soluble lysate of chromaffin granules with trypsin (0.05 mg/ml) for 10 min led to a decrease in the intensity of the chromogranin A band in electrophoretograms. Incubation of the insoluble residue of chromaffin granules under the same conditions caused a marked decrease in the intensity of the band that had the same mobility as chromogranin A, without causing any other change in the pattern. The protein content of the insoluble residue after incubation with trypsin decreased by 31%, but there was no significant change in the phospholipid content. The amino acid composition of the insoluble proteins of chromaffin granules is very different from that of chromogranin A (Helle & Serck-Hanssen, 1969; Winkler, 1969). Accordingly, if trypsin acted only on the chromogranin A present in the insoluble residue then a decrease of nearly one-third in the total protein would be accompanied by a change in the amino acid composition. That this did not occur is shown by the results of the analyses of the amino acid compositions of control and trypsin-treated membranes (Table 3). After hydrolysis of 32% of the protein (as determined from the total amount of amino acids in the hydrolysates) the amino acid composition was almost unchanged, showing that the bulk of the protein hydrolysed was not chromogranin A.

The effect of trypsin on the gel-electrophoresis pattern must therefore have another explanation. It is suggested that the highly acidic nature of chromogranin A causes it to stain more intensely than the other proteins so that it appears to make up a larger proportion of the insoluble proteins than it really does. This small amount of chromogranin A in the insoluble residue was found by immunochemical analysis to be more readily hydrolysed by trypsin than the bulk of the proteins: 68% of the chromogranin was destroyed under conditions which hydrolysed 32% of the protein.

The insoluble residue was also incubated with pronase (see the Methods section), and under these conditions 39% of the protein was solubilized but the phospholipids remained insoluble. The effect of pronase on the gel-electrophoretic pattern was similar to that of trypsin: the band corresponding to chromogranin A became very faint whereas there was only a relatively small decrease in intensity of the other bands.

DISCUSSION

There is no morphological evidence for the presence of insoluble material within adrenal chromaffin granules, as can be seen from the electron micrographs published by Diner (1967). The washed insoluble residue remaining after lysis of chromaffin granules by hypo-osmotic shock must therefore be composed of the membranes of the chromaffin granules.

The chromaffin granule membrane is characterized by its high content of lipids, by the presence of a Mg^{2+}-activated adenosine triphosphatase, cyto-

Table 3. *Amino acid composition of the total insoluble protein of chromaffin granules before and after tryptic hydrolysis*

Chromaffin granule membranes were incubated with trypsin as described in the Methods section. The amount of protein hydrolysed was 33% as determined by the biuret reaction and 32% as determined from the sum of the amounts of amino acids in the hydrolysate.

	Amino acid composition (%, w/w)	
	Total membrane proteins [mean ±S.D. ($n = 3$)]	After trypsin [mean ($n = 2$)]
Lys	6.13±0.04	5.94
His	2.80±0.05	3.20
NH$_3$	1.98±0.03	2.12
Arg	7.09±0.14	6.82
Asp	9.62±0.04	9.21
Thr	4.64±0.01	4.50
Ser	7.64±0.15	8.25
Glu	15.41±0.38	15.12
Pro	5.37±0.07	5.68
Gly	4.22±0.05	4.09
Ala	5.34±0.11	5.28
Cys	0.54±0.11	0.41
Val	5.00±0.09	4.74
Met	2.92±0.03	2.56
Ile	3.68±0.12	3.73
Leu	9.90±0.05	9.72
Tyr	4.05±0.10	4.12
Phe	5.51±0.17	5.53
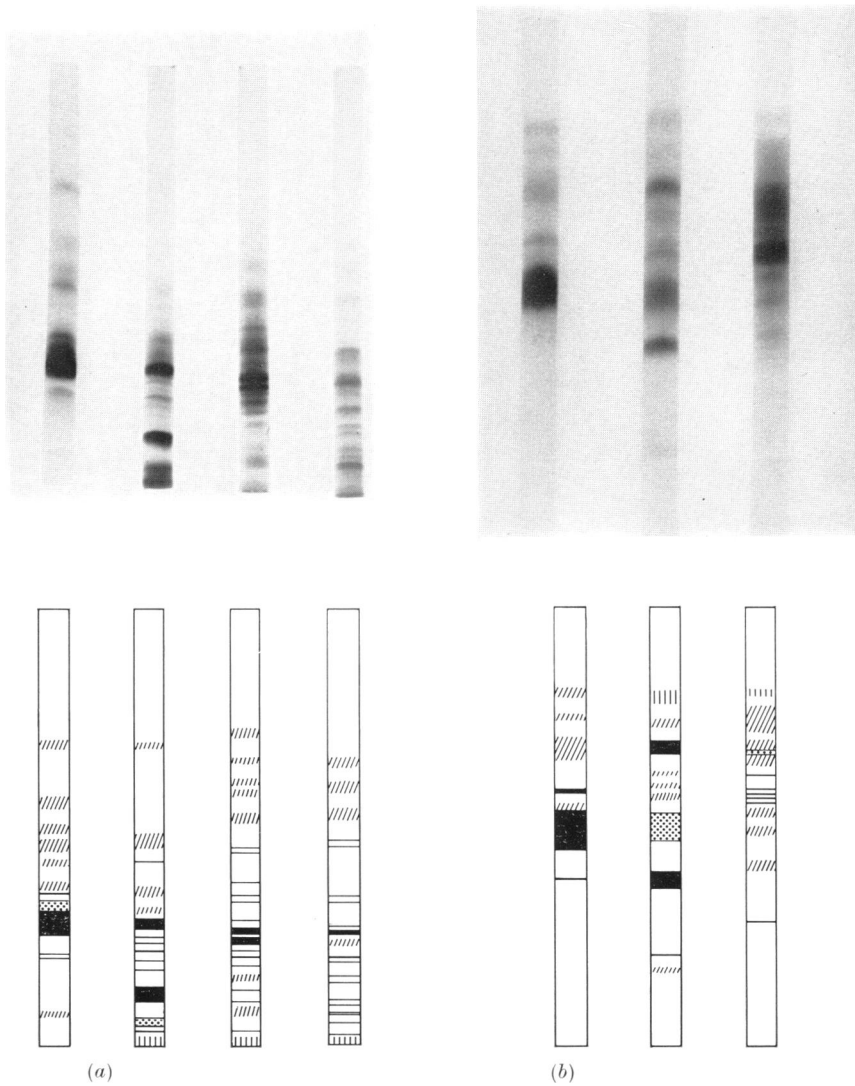
EXPLANATION OF PLATE I

Polyacrylamide disc electrophoresis of membranes from adrenal medulla. (*a*) Membranes were dissolved in phenol–acetic acid–urea and applied to gels equilibrated with the solvent (see the Methods section). The direction of migration was from the bottom to the top, which was the cathode. The stained gels are, from left to right: chromogranins (0.025 mg of protein); chromaffin granule membranes (0.09 mg); membranes of the mitochondrial fraction (0.11 mg); membranes of the microsomal fraction (0.11 mg). (*b*) Membranes were dissolved in alkaline sodium dodecyl sulphate, and the gels contained sodium dodecyl sulphate (see the Methods section). The direction of migration was from the bottom to the top, which was the anode. The stained gels are, from left to right: chromogranins (0.025 mg of protein); chromaffin granule membranes (0.09 mg); membranes of the mitochondrial fraction (0.11 mg).

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chrome b-559 and several other water-insoluble proteins. The finding that the membranes contain half the dopamine β-hydroxylase activity of the chromaffin granule confirms previous work (Belpaire & Laduron, 1968; Viveros et al. 1968) and raises the question whether the same molecular species of the enzyme is present in both parts of the chromaffin granule. The experiments in which the insoluble residue was washed five times show that the dopamine β-hydroxylase in the membranes is not just a contamination from the soluble lysate, but they do suggest that the chromogranin A in the insoluble fraction is derived from residual soluble lysate. This conclusion is supported by the experiments on the effect of trypsin on the amino acid composition and gel-electrophoretic pattern of the insoluble proteins. These observations do not support the suggestion (Helle & Serck-Hanssen, 1969) that chromogranin A is a major component of the insoluble proteins. The amino acid composition of the total membrane proteins is different from that of the chromogranins, as already reported (Winkler, 1969; Helle & Serck-Hanssen, 1969), being characterized by a smaller proportion of hydrophilic amino acids, in particular glutamic acid. This is further evidence that chromogranin A cannot be a major component of these proteins.

Most biological membranes contain, on a weight basis, more protein than lipid (for review see Korn, 1969). The chromaffin granule membrane appears to be unusual since the protein/lipid ratio is about 0.45. It is possible that this relative deficiency of protein is a reflection of the paucity of enzymes in the membrane, since myelin, which contains few enzymes, also has an unusually low protein/lipid ratio (0.25) (Korn, 1969).

In many endocrine and exocrine tissues secretion occurs by exocytosis, a process that involves a specific interaction between the membrane of the secretion granule and the plasma membrane followed by fusion of the two membranes. Do the secretion granules of these different tissues contain any common components that may be involved in exocytosis? In order to recognize such components, the secretion granules must first be isolated relatively free from contamination with other cell particles. This has only been achieved for a few secreting tissues (see review by Smith, 1968b).

Adrenal chromaffin granules are characterized by their high content of lysolecithin, and it has been suggested that this phospholipid is involved in the fusion of the chromaffin granule membrane with the plasma membrane (Blaschko et al. 1967b; Winkler et al. 1967). The ability of lysolecithin in vitro to bring about the fusion of membranes has been demonstrated by Howell & Lucy (1969). It is possible that the lysolecithin in chromaffin granules

is part of a specific lipoprotein, but our attempts to demonstrate such a lipoprotein by using detergents were not successful. Each of the detergents caused a preferential solubilization of protein, leaving the lipids in the insoluble residue, which suggests that the detergents destroyed the attractive forces between the proteins and the lipids. One of the detergents used, sodium dodecyl sulphate, solubilized 93% of the membrane proteins and this proved useful in the electrophoresis experiments.

Polyacrylamide-gel electrophoresis of chromaffin granule membrane dissolved with either sodium dodecyl sulphate or phenol–acetic acid–urea showed that the insoluble proteins were different from those of the mitochondrial and microsomal membranes. The chromaffin granule membrane contains one protein in particular which appears to be the main component, but which is by no means the major component of the other membranes. The evidence from gel electrophoresis, which shows that the membrane proteins from the different fractions are not identical, is in agreement with the different enzymic composition of the membranes.

The partial characterization of a specific protein of the chromaffin granule membrane opens up the possibility of studying dynamic aspects of membrane flow and vesiculation during secretion (Bennett, 1956; Palade, 1959). Is the membrane of the chromaffin granule synthesized together with the secretion products of the gland (hormones, ATP and chromogranins), or can the membrane be used more than once in a 'secretion cycle'?

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