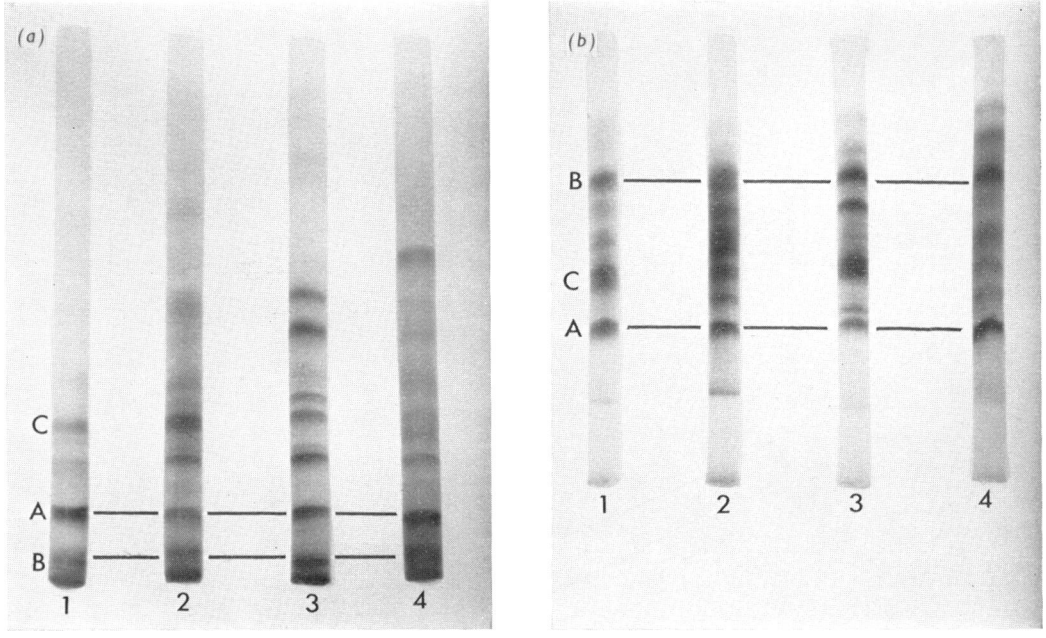
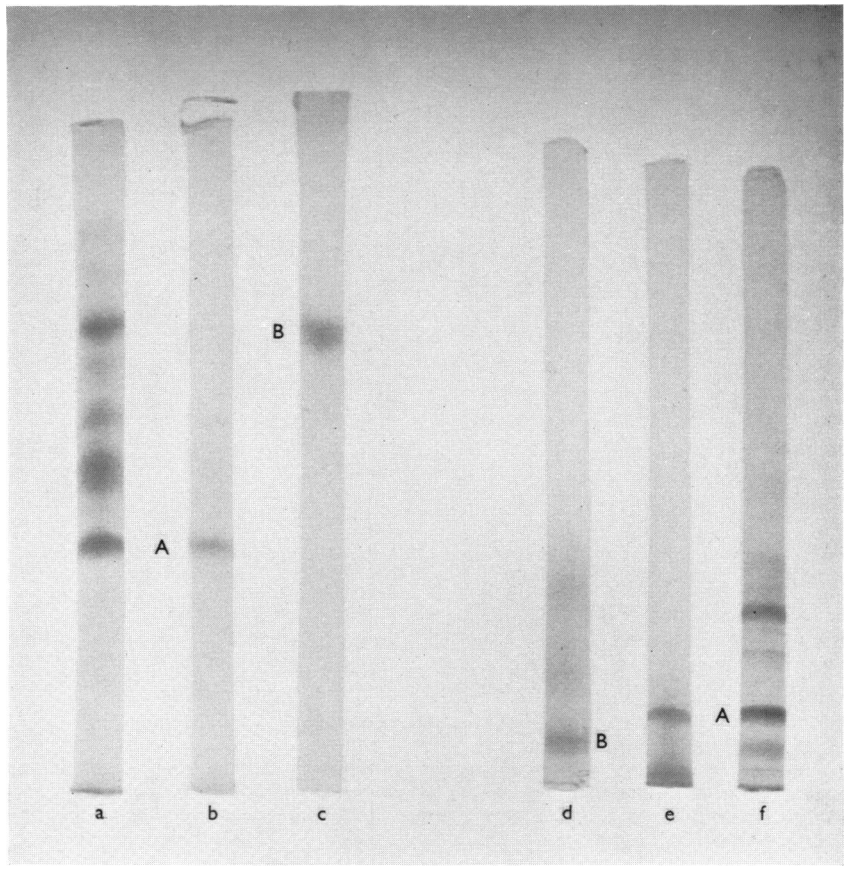


EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of membrane proteins from chromaffin granules. (*a*) Membranes were dissolved in phenol–acetic acid–urea and applied to gels equilibrated with the solvent. The direction of migration was from the bottom to the top, which was the cathode. (*b*) Membranes were dissolved in alkaline sodium dodecyl sulphate, and the gels contained sodium dodecyl sulphate. The direction of migration was from the bottom to the top, which was the anode. The membranes were obtained from chromaffin granules of ox (1), pig (2), horse (3) and a human phaeochromocytoma (4). Two major membrane proteins of bovine chromaffin granules are indicated by A and B. It is not certain whether the third major protein, indicated by C, is a genuine membrane component (see the Discussion section).



EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of the purified membrane proteins in two different buffer systems. Alkaline system: (a) total membrane proteins; (b) component A; (c) component B. Acid system: (d) component B; (e) component A; (f) total membrane proteins.

Biochem. J. (1971) **122**, 299–304
Printed in Great Britain

Membranes of Chromaffin Granules

ISOLATION AND PARTIAL CHARACTERIZATION OF TWO PROTEINS

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(*Received* 3 *December* 1970)

Membranes of chromaffin granules were isolated from the adrenal glands of four different species. The solubilized membrane proteins could be resolved into several bands by polyacrylamide-gel electrophoresis (alkaline and acid gel systems). Two major protein components appeared to be common to the chromaffin granule membranes of ox, horse, pig and man. The various membrane proteins of bovine chromaffin granules were separated by filtration on Sephadex G-200 in the presence of sodium dodecyl sulphate. Two major membrane proteins (A and B) were obtained in purified form. Treatment of protein A with 2-mercaptoethanol before electrophoresis resulted in two more rapidly migrating subunits, whereas protein B was unaffected by mercaptoethanol treatment. The amino acid compositions of the two purified proteins were determined. They are very similar to that of the total membrane proteins but significantly different from that of the chromogranins, the soluble proteins of chromaffin granules.

The hormones of the adrenal medulla are secreted by exocytosis (Banks & Helle, 1965; Sage, Smith & Kirshner, 1967; Schneider, Smith & Winkler, 1967; Poisner, Trifaró & Douglas, 1967; Diner, 1967), a process that has been shown to occur in many secreting cells (Stormorken, 1969). The final stage of exocytosis involves the fusion of the membrane of the chromaffin granule with the plasma membrane. A thorough understanding of this fusion process can only be obtained from a detailed knowledge of the various components of these membranes. In fact, the investigation of the lipids of chromaffin granules has already offered a first clue, since it was observed that these granules are characterized by a high concentration of lysophosphatidylcholine (lysolecithin) (Blaschko, Firemark, Smith & Winkler, 1967*a*; Winkler, Strieder & Ziegler, 1967*a*; Winkler & Smith, 1968; Trifaró, 1969). Lysophosphatidylcholine is a membrane-active compound that can not only lyse membranes, but can also bring about a fusion of membranes, as shown by Howell & Lucy (1969). It seems probable that lysophosphatidylcholine plays a decisive role in the fusion process that occurs during exocytosis in the adrenal medulla. However, the other major components of the interacting membranes, i.e. the proteins, are likely to participate. Whereas the soluble proteins of chromaffin granules, the chromogranins (Blaschko, Comline, Schneider, Silver & Smith, 1967*b*), have been isolated and well characterized (Helle, 1966*a*; Smith & Winkler, 1967*b*; Smith & Kirschner, 1967; Strieder, Ziegler, Winkler & Smith, 1968), much less is known about the insoluble membrane-bound proteins. We have investigated these membrane proteins by polyacrylamide-gel electrophoresis (Winkler, Hörtnagl, Hörtnagl & Smith, 1970). We now report the purification of two membrane proteins. A preliminary report of some of these results has been given (Winkler, 1971).

METHODS

Isolation of membranes from chromaffin granules. A large-granule fraction was obtained from homogenates of bovine adrenal medulla by differential centrifugation, as described by Winkler (1969). Chromaffin granules were isolated from this fraction by the simplified density-gradient method of Smith & Winkler (1967*a*). The same method was used for isolating chromaffin granules from horse and pig adrenal medullae. The granules from a human phaeochromocytoma were those from the case described by Winkler, Ziegler & Strieder (1967*b*), which had been kept in the deep-freeze.

Each pellet of chromaffin granules was resuspended in 2.0ml of hypo-osmotic buffer (tris–sodium succinate, pH 5.9 and I 0.005) to lyse the particles. The suspension was frozen and thawed and then centrifuged at 160000*g* for 45min. The sedimented membranes were washed four

times to remove the soluble components of chromaffin granules (see Winkler *et al.* 1970).

Polyacrylamide-gel electrophoresis. Disc electrophoresis was performed either in an acid medium containing phenol–acetic acid–urea or in an alkaline medium, in the presence of sodium dodecyl sulphate, as described in Winkler *et al.* (1970). In some experiments the membranes were dissolved in sodium borate–NaOH buffer, pH 9.6 and *I* 0.036, containing sodium dodecyl sulphate (1%, w/v) and 2-mercaptoethanol (2M) and incubated for 2h at room temperature before electrophoresis. For electrophoresis in the acid system an excess of phenol–acetic acid–urea was added to the solution after incubation.

Sephadex chromatography. Sephadex G-200 was swollen in sodium borate–NaOH buffer, pH 9.6 and *I* 0.036, containing sodium dodecyl sulphate (1%, w/v) for 3 weeks before use. Sodium azide (0.02%) was included to inhibit bacterial growth. For chromatography a column 118cm long × 2.3cm diam. was used. The hydrostatic pressure during filling and subsequent use was kept below 30cm H_2O and the flow rate ranged from 3 to 5ml/h. Fractions of volume about 2.5ml were collected, the elution of the proteins being followed by measuring the extinction at 280nm and by the Folin reaction (with bovine serum albumin as standard). Total phosphorus, derived from phospholipids, was determined by the method of Bartlett (1959).

Before chromatography isolated membranes of chromaffin granules (up to 15mg of protein) were resuspended in 1M-NaCl and then extracted with 2 vol. of diethyl ether (see Evans, Bandemer, Davidson, Heinlein & Vaghefi, 1968). This extraction was repeated once. The sedimented membrane residues were dissolved in up to 2ml of sodium borate–NaOH buffer containing sodium dodecyl sulphate (1%, w/v) and were left at room temperature for at least 5h. Chromatography was carried out at room temperature since the sodium dodecyl sulphate comes out of solution at lower temperatures.

For electrophoresis, pooled column fractions were dialysed for 5h at room temperature against the borate buffer to remove part of the sodium dodecyl sulphate and then concentrated by ultrafiltration at room temperature (Sober, Gutter, Wyckoff & Peterson, 1956).

Amino acid analysis. Protein samples obtained by Sephadex chromatography were concentrated as described above and then dialysed for 3 days against water at 2°C to remove most of the sodium dodecyl sulphate. After this prolonged dialysis the protein formed a precipitate. HCl was added to the suspension of protein to give a final concentration of 6M-HCl. The sample was hydrolysed for 17h at 110°C as described by Crestfield, Moore & Stein (1963). The amino acids in the hydrolysate were analysed with the Beckman model 120C amino acid analyser.

Enzyme assays. Dopamine β-hydroxylase and Mg^{2+}-stimulated adenosine triphosphatase activities were measured as described by Hörtnagl, Hörtnagl & Winkler (1969).

Materials. Sodium dodecyl sulphate was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., Sephadex G-200 and Blue Dextran were from Pharmacia Fine Chemicals, Uppsala, Sweden, sodium azide was from E. Merck A.-G., Darmstadt, Germany, and 2-mercaptoethanol was from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Gel electrophoresis of membrane proteins. The solubilized membrane proteins of bovine chromaffin granules can be resolved into several components by polyacrylamide-gel electrophoresis in both acid and alkaline gels (Winkler *et al.* 1970; see Plate 1). The proteins migrate differently in these two gel systems, but by using purified proteins (see below) the bands given by two main protein components (A and B) have been identified. The bovine proteins have now been compared with those of several other species (Plate 1). It is evident from Plate 1(*a*) and 1(*b*) that components corresponding to two major bovine proteins are present in the chromaffin-granule membranes from horse and pig medullae and in those from a human phaeochromocytoma.

In addition to these two proteins, there are several other components, one of which (C in Plate 1) appears to be common to the granule membranes of ox, horse and pig. However, it is not present in the proteins from phaeochromocytoma granules, since in the acid system (Plate 1*a*) no distinct band can be seen that migrates like component C. It has been shown (Winkler *et al.* 1970) that component C has the same mobility in both electrophoretic systems as chromogranin A, the major component of the soluble lysate. It has therefore been assumed that this band is due to contamination of the membranes by small amounts of soluble proteins. However, since this component has not yet been isolated and identified as chromogranin A, we prefer to term it component C.

Separation of membrane proteins by Sephadex chromotography. The proteins were separated by chromatography on Sephadex G-200 in the presence of sodium dodecyl sulphate. Before application to the column the membranes were extracted with ether. This procedure removed 76% of the phospholipids and 94% of the cholesterol, but did not change the electrophoretic behaviour of the membrane proteins. It appeared, however, that some of component C was removed preferentially during ether extraction. Since our aim was to isolate components A and B free from component C, this extraction was used as a routine; however, it should be mentioned that a similar elution profile from Sephadex G-200 was obtained with unextracted membranes.

The results of a typical experiment in which the eluate from a column of Sephadex G-200 was analysed for protein and phospholipid are given in Fig. 1. Most of the protein is eluted early, very near to the void volume. In a different experiment the void volume, as determined with Blue Dextran, was found to be 147.5ml; the elution volume of the main protein peak was 158ml.

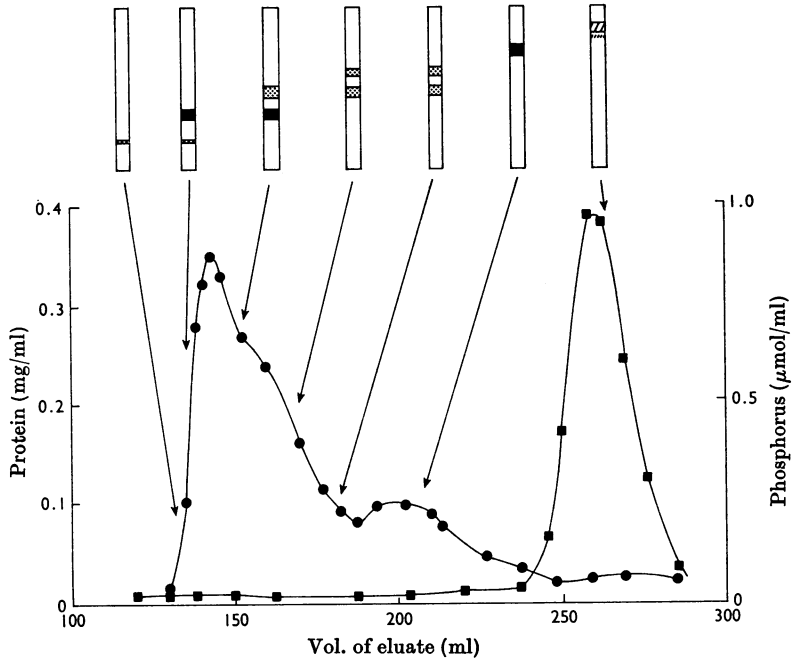

Fig. 1. Elution pattern of solubilized membranes from Sephadex G-200. For electrophoresis (alkaline gels) four to six of the eluted fractions were pooled and concentrated. The electrophoretic patterns obtained are shown above the elution curve. The direction of migration was from the bottom to the top, which was the anode. The arrows point to the regions in the elution curve from which the fractions were taken. ●, Protein; ■, phosphorus.

Disc electrophoresis in the alkaline medium of the various fractions from the column showed that a separation of the different proteins had been achieved. The electrophoretic patterns shown in Fig. 1 demonstrate that the proteins that are eluted early from the column migrate slowly in the gel. The first elution peak contains mainly component A, but a protein that migrates more slowly than component A is present in the ascending slope of this peak. The amount of this protein varied in different experiments and it was not always present (see, e.g., Fig. 1). It can be seen from Plate 1 that this protein corresponds to a very faint band in the electrophoretic pattern obtained from the total membrane proteins; the intensity of this band also varied from experiment to experiment.

The descending slope of the first peak, which exhibits a shoulder, contains component A plus component C. The small second peak contains component B, which seems to be well purified. The phospholipids are eluted after the proteins. In the electrophoresis system they are represented by the fastest-moving band.

The purified proteins obtained by gel filtration were used to identify the two major stained bands present in the acid and the alkaline polyacrylamide gels. This is shown in Plate 2. Component A, which moves more slowly than component C in the alkaline system, behaves in the same way in the acid system. However, the fast-moving component (B) of the alkaline system moves very slowly in the acid system, in fact even more slowly than component A.

Treatment with 2-mercaptoethanol. When the purified component A was treated with mercaptoethanol it was split into two subunits, which migrated faster on electrophoresis (see Fig. 2). The band designated A_1 in Fig. 2 always stained more strongly than the second, faster-moving, band.

The slow-moving component eluted as the first protein from Sephadex (see Fig. 1) was also treated with mercaptoethanol. This treatment resulted in the splitting of this component into subunits, which migrated identically with those formed from component A. It therefore seems likely that this protein is an aggregation product of component A. Treatment of purified component B with mercaptoethanol was without any effect.

Amino acid composition. Table 1 gives the amino acid compositions of the two purified proteins, as well as those of the total membrane proteins and

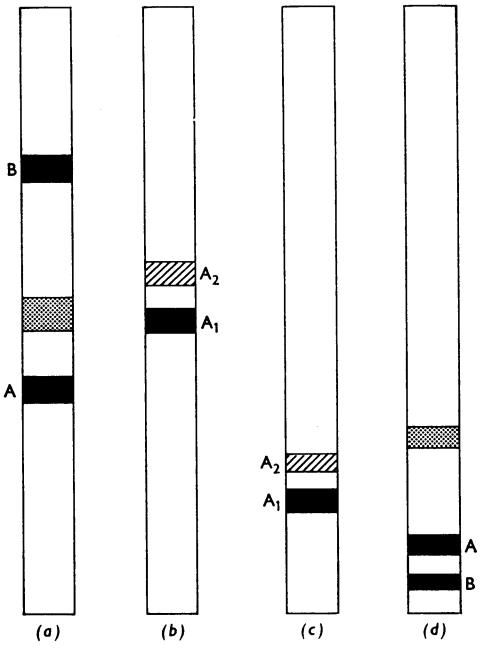

Fig. 2. Effect of 2-mercaptoethanol treatment on component A. Alkaline system: (*a*) total membrane proteins (only main components drawn); (*b*) component A after mercaptoethanol. Acid system: (*c*) component A after mercaptoethanol; (*d*) total membrane proteins (only main components drawn).

the total soluble proteins. The compositions of the two major membrane proteins are very similar to that of the total membrane proteins, whereas the composition of the soluble proteins, i.e. chromogranins, is significantly different (compare, e.g., 11.8–15.4% of glutamic acid in membrane proteins with 26.6% in the soluble proteins).

The amino acid composition of the proteins eluted from Sephadex between components A and B (including component C; see Fig. 1) was also determined. Their glutamic acid content was 13.4%.

Identification of enzymes. The membranes of chromaffin granules contain an Mg^{2+}-stimulated adenosine triphosphatase, dopamine β-hydroxylase and cytochrome *b*-559. Unfortunately, it has proved to be impossible to measure adenosine triphosphatase and dopamine β-hydroxylase activities in the fractions eluted from the Sephadex column, since both enzymes are inactive in the presence of sodium dodecyl sulphate.

Cytochrome *b*-559 has a characteristic absorption spectrum (Ichikawa & Yamano, 1965; Banks, 1965). The spectra of the fractions eluted from Sephadex were therefore determined. The only fractions exhibiting the characteristic extinction peak at about 405nm were those containing the phospholipids but little protein. This extinction peak was shifted to 416nm after the addition of a trace amount of sodium dithionite, which confirms that it was due to the cytochrome (see Banks, 1965). Gel electrophoresis of these fractions demonstrated a faint protein band (see Fig. 1) that may represent the cytochrome.

DISCUSSION

The solubilized membrane proteins of bovine chromaffin granules can be resolved into several bands by polyacrylamide-gel electrophoresis in two different buffer systems (Winkler *et al.* 1970). Two major protein components were demonstrated. When solubilized membranes of the chromaffin granules from several other species (horse, pig and man) were subjected to electrophoresis in these systems, strongly staining protein bands, which migrated to the same position as those from bovine chromaffin granules, were observed. It therefore seems likely that identical or very similar proteins are the major components of the granule membranes of several species. It is interesting to compare these results with those obtained from the soluble proteins (chromogranins) of chromaffin granules. Common components of soluble proteins were only found in ox, horse and pig granules (Winkler, Ziegler & Strieder, 1966; see also Helle, 1966*b*; Hopwood, 1968), but not in those from a phaeochromocytoma (Strieder *et al.* 1968).

In addition to the two major proteins, several other protein bands were observed after gel electrophoresis. One of them, component C, migrates in two electrophoretic systems like chromogranin A (Winkler *et al.* 1970). Component C was not isolated in the present study, since Sephadex chromatography did not separate it well from the other proteins. Thus an amino acid analysis could only be obtained for a fraction containing other proteins besides component C. This fraction had a glutamic acid content of 13.4% whereas chromogranin A has one of 26.6%. This result demonstrates that chromogranin A can be only a very minor component of this fraction, despite the fact that a well-stained band corresponding to chromogranin A is present in the electrophoretic pattern. It has been suggested that chromogranin A stains more strongly than the membrane proteins (Winkler *et al.* 1970), which would explain the present finding. In any case, it is clear that other proteins beside chromogranin A are eluted from the Sephadex column between components A and B.

The membranes of chromaffin granules contain cytochrome *b*-559 (Ichikawa & Yamano, 1965; Banks, 1965), and adenosine triphosphatase (Banks, 1965; Kirshner, Kirshner & Kamin, 1966; Winkler

Table 1. *Amino acid compositions of proteins from chromaffin granules*

The composition is given in g of amino acid/100g of protein (means ±S.D., $n = 3$ for component A and $n = 5$ for component B). The values for the total membrane proteins were taken from Winkler *et al.* (1970) and those for the total soluble proteins (chromogranins) from Strieder *et al.* (1968). Component A was obtained by concentrating those fractions forming the ascending slope of the first peak eluted from the Sephadex column. Component B was obtained from the fractions corresponding to the second peak (see Fig. 1).

	Amino acid composition (g/100g of protein)			
	Component A	Component B	Total membranes	Chromogranins
Lys	5.6±0.5	7.0±0.8	6.1	8.6
His	3.1±0.2	3.2±0.2	2.8	3.6
Arg	7.9±0.2	7.4±0.3	7.1	10.7
Asp	9.4±0.1	8.9±0.2	9.6	7.7
Thr	4.9±0.2	5.2±0.3	4.6	2.3
Ser	5.4±0.1	5.9±0.4	7.6	5.2
Glu	13.5±0.1	11.8±0.5	15.4	26.6
Pro	6.0±0.3	5.2±0.6	5.4	8.0
Gly	4.4±0.1	4.7±0.2	4.2	3.8
Ala	5.3±0.1	5.8±0.2	5.3	4.2
Cys	1.1±0.4	1.2±0.2	0.5	0.6
Val	5.3±0.4	5.2±0.5	5.0	2.7
Met	2.3±0.1	2.6±0.4	2.9	1.8
Ile	4.2±0.2	3.7±0.4	3.7	1.0
Leu	10.6±0.2	10.0±0.8	9.9	6.8
Tyr	4.7±0.1	5.6±0.7	4.1	2.2
Phe	6.2±0.3	6.5±0.2	5.5	2.3

et al. 1970) and dopamine β-hydroxylase (Viveros, Arqueros & Kirshner, 1968; Belpaire & Laduron, 1968; Winkler *et al.* 1970). In the present study cytochrome *b*-559 could be correlated with a minor protein component of the membranes. An identification of adenosine triphosphatase and dopamine β-hydroxylase has not yet been achieved, since the activities of these two enzymes were lost when sodium dodecyl sulphate was present. However, it should be mentioned that dopamine β-hydroxylase has been isolated from bovine adrenal medulla (see Kaufman & Friedman, 1965), but it is not known whether this purified enzyme was derived from the granule membranes or from the soluble content, or from both.

Sephadex-gel filtration of the membrane solubilized by sodium dodecyl sulphate has allowed us to separate the various proteins preparatively. Component B appeared to be well purified, migrating as a single band in gel electrophoresis. Preparations of component A usually gave a slowly migrating band in addition to the main component on electrophoresis. It seems likely that this additional component is only a large-molecular-weight aggregation product of component A, since treatment with mercaptoethanol resulted in the formation of two identical subunits irrespective of whether component A or the slowly migrating component was treated. A preparative separation of these two subunits seems possible, since preliminary chromatography experiments in the presence of 0.2M-mercaptoethanol gave a partial separation of these two components.

Proteins are eluted from columns of Sephadex according to their size, large molecules being eluted first, whereas in the polyacrylamide gel large molecules are retarded. In agreement with this there is some correlation between the results obtained from Sephadex-gel filtration and those from gel electrophoresis. Component A was eluted first from the Sephadex column, indicating a large size, and it migrated slowly in both electrophoretic systems. Component B appears to be a small molecule because of its behaviour both on Sephadex and in the alkaline electrophoretic system. However, a different result was obtained in the acid electrophoretic system, where component B moved more slowly than the other proteins. Two explanations can be offered: in the acid system component B may aggregate to give a rather large molecule. This, however, seems unlikely, since phenol–acetic acid–urea should prevent aggregation and because mercaptoethanol was without effect on this protein. The second possibility is that component B is in a very contracted state in the alkaline system, but in a very expanded form in the acid system. This would imply that these two membrane proteins, which have about the same amino acid composition, must have a completely different tertiary molecular structure. Is that in any way connected with a different localization of these proteins within the membranes? It will be interesting to see whether

reconstitution experiments, i.e. a recombination of membrane lipids with the membrane proteins, will offer a clue in this direction.

It has already been mentioned that the membranes of chromaffin granules are involved in the fusion process occurring during exocytosis. We are still a long way off an understanding of this process at the molecular level. However, a further characterization of protein components that are likely to participate in this process has now been achieved.

We thank Dr F. Spöttl for his advice on the Sephadex experiments. This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung (Austria).

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