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Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114

Application to subcellular fractions of the adrenal medulla

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After solubilization with the detergent Triton X-114, membrane proteins may be separated into three groups: if the membrane is sufficiently lipid-rich, one family of hydrophobic constituents separates spontaneously at low temperature; warming at 30 °C leads to separation of a detergent-rich phase and an aqueous phase. Using the chromaffin-granule membrane as a model, we found that many intrinsic membrane glycoproteins are found in the latter phase, probably maintained in solution by adherent detergent. They precipitate, however, when this is removed by dialysis, leaving in solution those truly hydrophilic proteins that were originally adhering to the membranes. We have used this method with mitochondria, and with Golgi- and rough-endoplasmic-reticulum-enriched microsomal fractions: it has proved to be a rapid and convenient method for effecting a partial separation of proteins from a variety of different membranes.

INTRODUCTION

There are several well-known problems associated with the analysis of membrane proteins: (1) in many cases it is difficult to obtain pure membrane fractions; (2) cytosolic, luminal or matrix proteins are inevitably associated with the membranes; and (3) no simple method exists to identify intrinsic proteins in the presence of such contamination. Electrophoretic analysis of membrane proteins has been improved by the introduction of two-dimensional separations, and the transfer of separated polypeptides to cellulose nitrate sheets has enhanced the sensitivity of antibody and lectin overlays. Using these techniques to analyse subcellular fractions of the adrenal medulla, we have developed some general methods which largely overcome these problems.

The methods were developed from the technique used by Bordier (1981) to separate mixtures of hydrophobic and hydrophilic proteins. The detergent Triton X-114, which forms clear micellar solutions in water at low temperatures, undergoes a phase separation owing to the formation of large micellar aggregates on warming above 20 °C. It is a good detergent for solubilizing membranes (Egan *et al.*, 1976) and this reversible phase-separation property can thus be used to separate intrinsic membrane proteins from the more hydrophilic proteins associated with membrane preparations. We report here that use of Triton X-114 allows the separation of up to four quite distinct families of proteins from purified membrane preparations. In particular, we have applied this method for a new analysis of the proteins associated with preparations of membranes of adrenal-medullary secretory granules (chromaffin granules).

Several detailed analyses of chromaffin-granule proteins have been published. Abbs & Phillips (1980), studying the topographical distribution of proteins by using one-dimensional electrophoresis and various protein-labelling techniques, showed that most of the Coomassie Blue-stained polypeptides were exposed to the outer (cytosolic) side of the secretory-granule membrane. They also confirmed the finding (Huber *et al.*, 1979) that the carbohydrate structures of membrane glycoproteins were on the inner or matrix face of the membrane. Subsequently more detailed electrophoretic analyses of the membrane and soluble matrix glycoproteins have been presented (Gavine *et al.*, 1984; Apps *et al.*, 1985). Techniques based on the use of Triton X-114 have enabled us to enhance the resolution obtained in previous studies, and seem also to be of wide applicability.

MATERIALS AND METHODS

Materials

Bovine adrenal glands were obtained from a local slaughterhouse and were placed on ice within about 20 min of slaughter. Triton X-114 was obtained from Fluka A.G., Buchs, Switzerland. Ampholytes were either from Bio-Rad Laboratories, Watford, Herts., U.K., or L.K.B., South Croydon, Surrey, U.K. Other electrophoresis materials were from B.D.H., Poole, Dorset, U.K.; cellulose nitrate sheets were from Schleicher and Schüll, Dassel, West Germany. A cholesterol assay kit was obtained from Boehringer Mannheim, Lewes, Sussex, U.K. Other biochemicals, including lectins, were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The lectins were iodinated as described by Gavine *et al.* (1984), to final specific radioactivities of 2×10^6–2×10^7 Bq/mg of protein ($Na^{125}I$ was obtained from Amersham International, Amersham, Bucks., U.K.). *NN'*-Dicyclohexyl[^{14}C]carbodi-imide was obtained from C.E.A., Gif-sur-Yvette, France. The antiserum to mitochondrial F_1-ATPase from bovine heart was described by Apps & Schatz (1979). The pH of 1 M-Hepes solutions was adjusted with NaOH.

Methods

Membrane preparation. Chromaffin granules were isolated from bovine adrenal glands in 0.3 M-sucrose containing 20 mM-Hepes, pH 7.0, and 0.2 mM-phenyl-

methanesulphonyl fluoride at 0 °C. After purification through buffered 1.8 M-sucrose, the contents were released by osmotic lysis and the membranes were purified over a 1.0 M-sucrose step gradient (Apps & Schatz, 1979). The membranes were washed and resuspended in 20 mM-Hepes, pH 7.0, containing 1 mM-dithiothreitol and 1 mM-EDTA, and were used either fresh or after storage at −20 °C. Mitochondrial membranes, and microsomal fractions enriched in the Golgi complex or rough endoplasmic reticulum, were prepared as described by Gavine *et al.* (1984). The lysate from the granules was supplemented with fresh 0.2 mM-phenylmethanesulphonyl fluoride and was dialysed against 1000 vol. of 1 mM-EDTA/1 mM-Hepes, pH 7.0. It was freeze-dried, dissolved in distilled water, centrifuged for 2 h at 150000 *g* at 4 °C, filtered through a 0.45 μm cellulose nitrate filter and stored at −20 °C.

The procedure of Howell & Palade (1982) was followed when membranes were washed with 0.2 M-Na_2CO_3.

Triton X-114 treatment. Triton X-114 was precondensed as described by Bordier (1981). It was used as a 10% (w/v) stock solution. Membranes were washed once in 0.15 M-NaCl/10 mM-Tris/HCl, pH 7.6 (Tris/salt buffer), then resuspended by homogenization. Triton X-114 was added at 0 °C, so that final concentrations were 2% (w/v) detergent and 4 mg of protein/ml. The solubilized membranes were placed on ice: after about 1 min a white precipitate formed. After a further 4 min, this was removed by centrifugation for 30 min at 58000 *g* in a Beckman SW50.1 rotor. The pellet was washed twice at 0 °C by resuspension to its original volume in Tris/salt buffer, first in the presence of 2% Triton X-114, and then in its absence.

The supernatant left after removal of this phospholipid-rich material was layered over a cushion of 0.25 M-sucrose in Tris/salt buffer (2 ml) containing 0.06% Triton X-114, in a conical glass centrifuge tube. After 5 min at 30 °C the solution became turbid. It was centrifuged for 5 min at 2500 *g* at room temperature in a swing-out rotor of a bench centrifuge. A detergent-rich phase collected under the cushion of sucrose.

The aqueous phase above the sucrose was removed and made 0.5% (w/v) with Triton X-114, mixed well and placed on ice for dissolution of the detergent. It was then replaced over the sucrose cushion used above, warmed at 30 °C for 5 min, and the tube was re-centrifuged. The resulting supernatant was cooled at 0 °C, made 2% (w/v) in Triton X-114, mixed well and then incubated for 5 min at 30 °C. It was centrifuged to remove the condensed detergent, which was discarded.

After washing of the centrifuge tube and the top of the sucrose cushion twice with Tris/salt buffer, the viscous detergent phase was diluted to its original volume with Tris/salt buffer and cooled to 0 °C to redissolve the detergent. This was warmed at 30 °C and the detergent-rich phase re-centrifuged through 0.25 M-sucrose. The resulting washed detergent-rich phase was collected, diluted with 1 or 2 vol. of Tris/salt buffer and stored at −20 °C.

Dialysis of the aqueous phase. Residual Triton X-114 (0.06%, as found by analysis) was removed from the aqueous phase by dialysis at 4 °C against Tris/salt buffer in which was dispersed 1% (w/v) Amberlite XAD-2. After 1–2 days the medium was replaced, and dialysis was continued. The dialysed fraction was then diluted with 3 vol. of water and centrifuged for 2 h at 165000 *g*; this yielded a pellet which was washed and analysed separately from the supernatant, which was passed through a nitrocellulose filter (0.45 μm pore size). More than 90% of the Triton X-114 was removed by this procedure.

Electrophoresis. Samples for one-dimensional electrophoresis were delipidated in 10–75 vol. of acetone/ethanol (1:1, v/v) in silicone-treated Corex tubes in an ice/salt bath. They were then solubilized in sample buffer containing 5% (w/v) SDS and 5% (w/v) β-mercaptoethanol and analysed by electrophoresis in exponential (7–15 or 8–15%, w/v) polyacrylamide slab gels containing SDS as previously described (Gavine *et al.*, 1984). Isoelectric focusing for two-dimensional electrophoresis (O'Farrell, 1975) was on gels (11 cm × 0.3 cm) containing 4.5% (w/v) acrylamide, 0.06% (w/v) bisacrylamide, 9.5 M-urea, 2% (w/v) Triton X-100 and 2% (v/v) ampholytes (Bio-Rad 3–10 and LKB 9–11; 5:1, v/v). Samples were loaded at the basic (negative) end and the gels were focused for 30 min at 100 V, then at 200 V until the current was less than 0.5 mA per tube; the voltage was then increased to 400 V for 15–20 h, followed by 0.5 h at 800 V. Samples (200–300 μg of protein) were prepared by freeze-drying and solution in 9.5 M-urea containing 2% ampholytes, 2% Triton X-100, 5% β-mercaptoethanol and 15–30 μg of Bromophenol Blue/ml. They were clarified by centrifugation: any floating lipid was discarded. Samples in Tris/salt buffer were desalted by using small columns of Biogel P-6DG.

After electrophoresis, radioactive gels were fixed in methanol/acetic acid/water (2:1:7, by vol.) for 30 min, washed briefly in water, soaked in 1 M-sodium salicylate for 1 h, dried on Whatman 3 MM paper, then autoradiographed at −70 °C for 7–14 days.

Electrophoretic transfer of proteins to cellulose nitrate sheets and immunoblotting were performed by the method of Towbin *et al.* (1979), with the solutions previously described (Gavine *et al.*, 1984). After a wash in Tris/salt buffer containing 3% (w/v) bovine serum albumin for 60 min, the sheets were incubated in Tris/salt buffer containing 5% (v/v) inactivated horse serum and antiserum, usually diluted 100-fold. After five washes with Tris/salt buffer, they were treated with ^{125}I-protein A (4 kBq/ml). After five further washes in Tris/salt buffer, one containing 0.1% Triton X-100, they were dried and autoradiographed for 1–7 days by using Agfa–Gevaert Curix RP-1 film.

Decoration with lectins. Cellulose nitrate replicas of gels were washed with periodate-treated bovine serum albumin (3%, in Tris/salt buffer) for 4 h. For this purpose 4% albumin in 0.1 M-acetic acid was treated with 10 mM-$NaIO_4$ for 6 h, at 20 °C. Glycerol was then added to 10 mM and the solution was dialysed against Tris/salt buffer containing NaN_3 (0.5 mg/ml).

The sheets were then washed five times in Tris/salt buffer and incubated overnight with ^{125}I-labelled lectin (10 kBq/ml). They were then washed five more times, the penultimate wash containing 0.1% Triton X-100 (this was omitted when wheat-germ agglutinin had been used). Control replicas were incubated with ^{125}I-lectin in the presence of the appropriate hapten sugar at 100 mM. Sheets were then autoradiographed for 3–21 days.

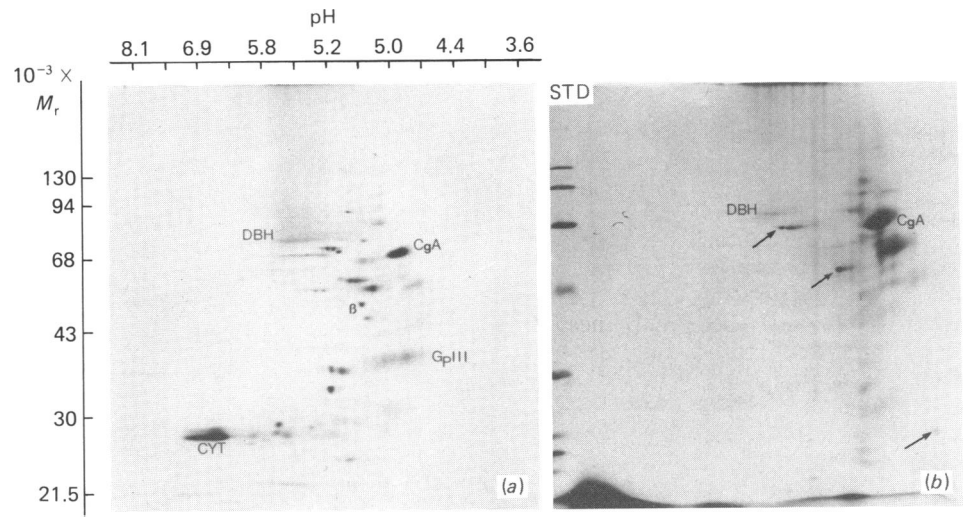

Fig. 1. Two-dimensional electrophoretic analysis of chromaffin-granule membrane proteins

(*a*) Complete membranes; (*b*) proteins removed by washing with Na_2CO_3. Arrows in (*b*) indicate proteins not found in the lysate of chromaffin granules, as identified from Fig. 1 of Apps *et al.* (1985). Abbreviations: DBH, dopamine β-hydroxylase; CgA, chromogranin A; β, β-subunit of F_1-ATPase; G_PIII, glycoprotein III of Huber *et al.* (1979); CYT, cytochrome b_{561}; STD, standard proteins (one dimension): β-galactosidase (M_r 130000); phosphorylase (M_r 94000); bovine serum albumin (M_r 68000); ovalbumin (M_r 43000); carbonic anhydrase (M_r 30000); trypsin inhibitor (M_r 21500); myoglobin (M_r 17200); lysozyme (M_r 14300).

Other methods. Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard. When detergent was present in samples, the method of Hartree (1972) was used. Lipid phosphorus determination was by a method based on that of Bartlett (1959). Triton was assayed by the method of Garewal (1973).

Chromaffin-granule membranes and mitochondrial membranes (4 mg of protein/ml) were covalently labelled with radioactive dicyclohexylcarbodi-imide by incubation in a solution containing 10 mM-Hepes, pH 7.4, 0.5 mM-EDTA, 10 mM-ATP and 20 μM-*NN'*-dicyclohexyl[^{14}C]-carbodi-imide (sp. radioactivity 1.85 GBq/mol). The samples were rotated slowly at 4 °C for 15 h, then washed in Tris/salt buffer.

Bovine heart F_1-ATPase, type I, was prepared by the method of Fisher *et al.* (1981).

RESULTS

Removal of adherent soluble proteins

The secretory granules of the adrenal medulla (chromaffin granules) are the best-characterized subcellular fraction of this tissue. Preparations of their membranes are, however, always contaminated by secretory proteins, in particular chromogranin A, as well as by cytosolic and extracellular components. Washing membranes with 0.2 M-Na_2CO_3 breaks open vesicular structures and removes extrinsic contaminants (Howell & Palade, 1982; Higgins, 1984).

A two-dimensional electrophoretic separation of chromaffin-granule membrane proteins is shown in Fig. 1(*a*). The major proteins of the membrane preparation are cytochrome b_{561} (M_r 26000, pI 6.2), dopamine β-hydroxylase (M_r 75000, pI 5.4; this protein enters the second dimension poorly and is always revealed as a smear in the top part of the gel, and chromogranin A, the major matrix protein (M_r 70000, pI 4.9). One of the major glycoproteins (M_r 37000; glycoprotein III of Huber *et al.*, 1979) can also be seen. A distinct subset of proteins, almost identical with those released by lysis of intact granules (Apps *et al.*, 1985), is removed by the Na_2CO_3-washing procedure (Fig. 1*b*). These proteins have been characterized by immunological techniques and have been shown to contain chromogranins A and B, and the products of their proteolysis (Fischer-Colbrie & Frischenschlager, 1985), and a small amount of the matrix enzyme dopamine β-hydroxylase. There are, however, a few non-matrix proteins (shown in Fig. 1*b*), which are almost certainly adhering to the cytosolic surface of the granule membrane: among these are the β-subunit of mitochondrial F_1-ATPase (M_r 50000, pI 5.1), and three components that we also find on gels of endoplasmic reticulum and Golgi fractions. After two or three cycles of washing, depletion of these components from the membrane preparation is almost complete; a trace of chromograin A remains as the most conspicuous contaminant. Having identified this set of proteins in this way, we did not normally prewash membranes before using the phase-separation procedure.

Solubilization and temperature-induced phase separation with Triton X-114

Triton X-114, when used at a detergent:protein ratio of 5:1 at 0 °C, was a good agent for solubilizing chromaffin-granule membrane proteins. However, unlike solubilization with Triton X-100 or octaethyleneglycol dodecyl ether (Apps *et al.*, 1980), a white precipitate spontaneously separated from the resulting solution. This effect was not pH-dependent; the pellet was composed of aggregates of detergent, phospholipid and cholesterol, together with some very hydrophobic proteins (approx. 10% of the chromaffin-granule membrane protein). We refer to the pellet as the phospholipid-rich phase.

On warming at 30 °C, the supernatant became turbid

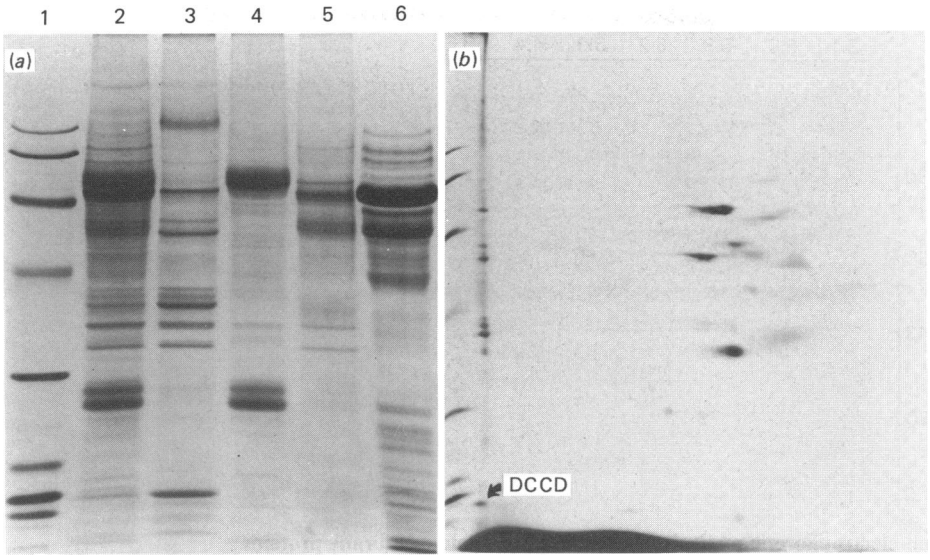

Fig. 2. Phase separation of chromaffin-granule membrane proteins

(*a*) Proteins from the Triton X-114 phase-separation procedure were analysed under reducing conditions on an 8–15%-polyacrylamide gel. Track 1, standard proteins, as in Fig. 1. Track 2, complete chromaffin-granule membranes. Track 3, phospholipid-rich phase. Track 4, detergent-rich phase. Track 5, aqueous phase. Track 6, chromaffin-granule lysate (matrix) proteins; the major band is chromogranin A (M_r 70000). (*b*) Two-dimensional analysis of the phospholipid-rich phase. Abbreviation: DCCD, polypeptide that binds [^{14}C]dicyclohexylcarbodi-imide (Sutton & Apps, 1981).

as large micellar aggregates formed (Bordier, 1981). A detergent-rich phase was separated from an aqueous phase by low-speed centrifugation through sucrose; it contained about 50% of the membrane protein, leaving about 30–40% in the aqueous supernatant.

Analysis showed that the phospholipid/protein and cholesterol/protein ratios were 3 and 4 times higher, respectively, in the phospholipid-rich phase than in the detergent-rich phase; neither phospholipid nor cholesterol was detected in the aqueous phase. Some 95% of the Triton X-114 was recovered in the detergent-rich phase.

Samples of the three phases were examined by polyacrylamide-gel electrophoresis (Fig. 2*a*). Quite distinct families of proteins are recovered in the three phases, with little overlap between them. This is summarized in Table 1, which gives the distribution of 24 major polypeptides, with M_r values between 104000 and 13000.

Characterization of chromaffin-granule membrane proteins

In order to characterize the three phases more completely, we analysed each by two-dimensional electrophoresis, identifying proteins where possible by immunological or other methods. We also treated transfers of two-dimensional gels with radioiodinated lectins in order to identify glycoproteins: these are major constituents of chromaffin-granule membranes, but, with a few exceptions, they cannot be observed as Coomassie Blue-stained bands (Abbs & Phillips, 1980; Gavine *et al.*, 1984), so we discuss them separately below.

A two-dimensional gel of the proteins of the phospholipid-rich phase is shown in Fig. 2(*b*). Among these proteins is found a very hydrophobic low-M_r polypeptide that reacts with dicyclohexylcarbodi-imide (Table 1; Sutton & Apps, 1981), which is one component

Table 1. Distribution of major chromaffin-granule membrane polypeptides

Identification is based on immune replicas, labelling with [^{14}C]dicyclohexylcarbodi-imide (DCCD), and previous work on matrix components (Apps *et al.*, 1985). Key: +, major location; ±, minor location.

	Phase			
$10^{-3} \times M_r$	Phospho-lipid	Deter-gent	Aqueous	Identi-fication
104			+	Matrix
93			+	Matrix
78		+	+	Dopamine
75		+		β-hydroxylase
72	+			
70			+	Chromogranin A
68			+	
65		+		
62		+		Matrix
58	±		+	
56			+	Matrix
53	+			
50		+		
47	+			
40	+			
39	+			
37	+			
34	+	±	±	
31	+		±	
26		+		Cytochrome b_{561}
24		+		
17	+			DCCD-reactive
16	+			
13	+			

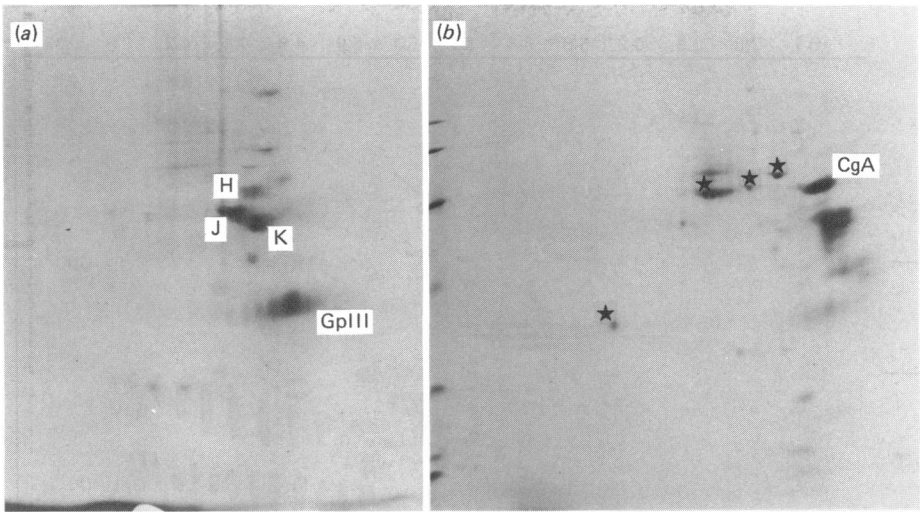

Fig. 3. Chromaffin-granule membrane proteins in the aqueous phase

(*a*) Proteins precipitated on removal of detergent; (*b*) proteins remaining in solution after removal of detergent. The proteins labelled in (*a*) are major glycoproteins that stain with Coomassie Blue (cf. Fig. 5); stars in (*b*) denote proteins that are not components of the granule matrix. Abbreviation: CgA, chromogranin A. Standard proteins were as in Fig. 1.

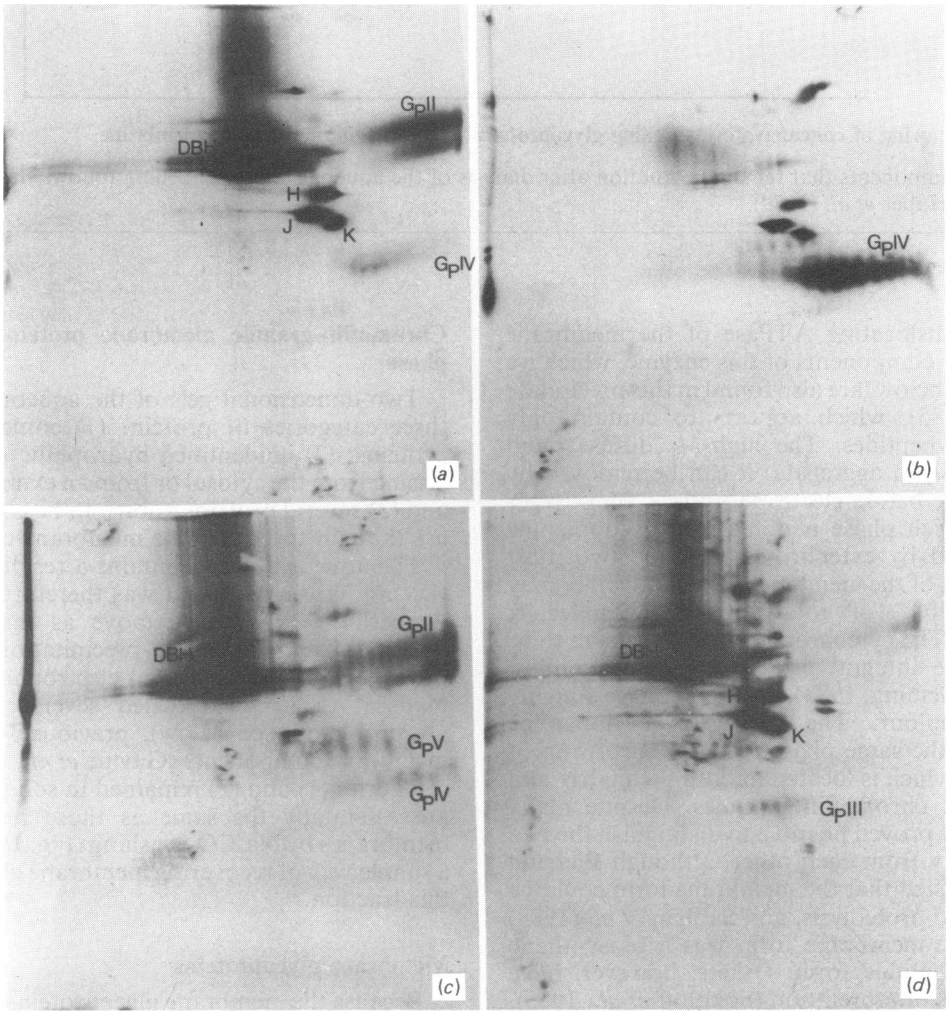

Fig. 4. Concanavalin A overlays of two-dimensional electrophoretograms of chromaffin-granule membrane proteins

(*a*) Whole membranes; (*b*) phospholipid-rich phase; (*c*) detergent-rich phase; (*d*) whole aqueous phase. Abbreviation: DBH, dopamine β-hydroxylase. Labelling of other glycoproteins refers to Fig. 5.

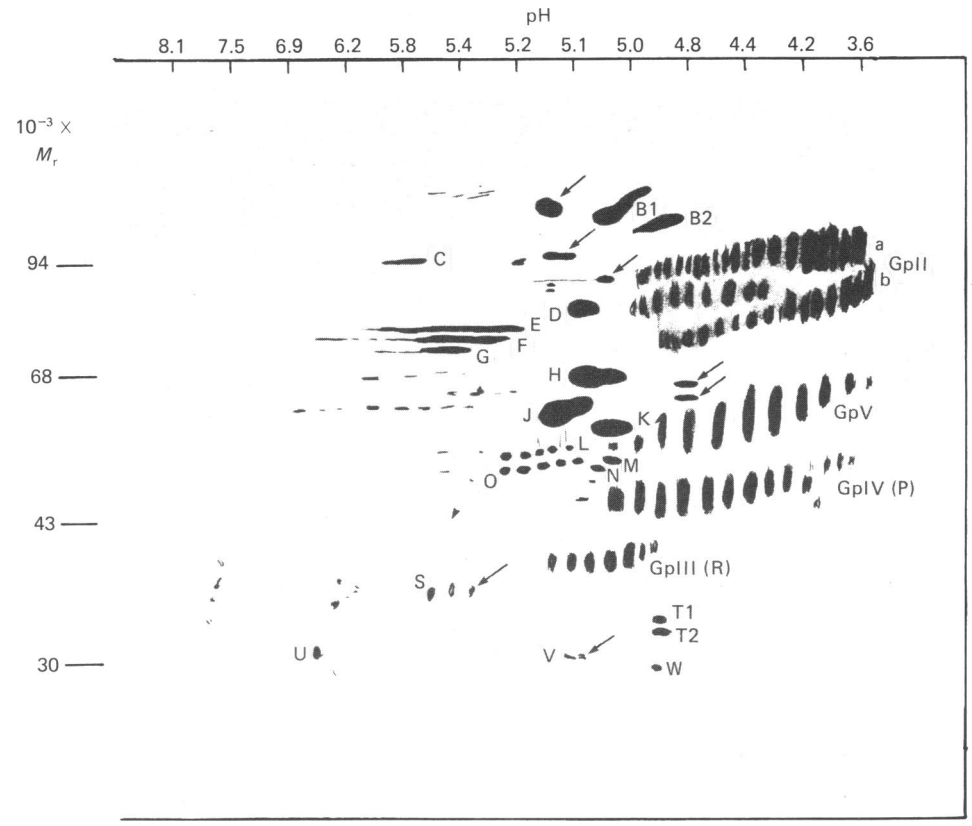

Fig. 5. Composite drawing of concanavalin A-binding glycoproteins of the chromaffin-granule membrane

Arrows denote components that remain in solution after dialysis of the aqueous phase. Labelling follows that used by Gavine *et al.* (1984) and Huber *et al.* (1979).

of the proton-translocating ATPase of the membrane (ATPase I). Other components of this enzyme, which we discuss separately below, are also found in this precipitate (Percy *et al.*, 1985), which appears to contain only hydrophobic polypeptides. The high-M_r diffuse band seen in Fig. 2(*a*) is an aggregate: it can be removed by heating the sample before electrophoresis.

The detergent-rich phase is dominated by dopamine β-hydroxylase and by cytochrome b_{561}, the two most abundant proteins of the membrane, which together may comprise over 40% of total protein (Winkler & Westhead, 1980). The cytochrome, which is known to be a trans-membrane integral protein (Abbs & Phillips, 1980; Duong & Fleming, 1984), gives this phase a highly distinctive red colour. The presence of dopamine β-hydroxylase in the same phase is rather surprising: it is a glycoprotein which is located in both the matrix and the membrane of chromaffin granules. Despite many attempts it has not proved possible to distinguish the two forms convincingly from each other, although Bjerrum *et al.* (1979) suggested that the membrane form could be solubilized by mild proteolysis, and Sabban *et al.* (1983) suggested that the membrane form was a biosynthetic precursor of the soluble form. Others, however, have disagreed with this interpretation (Sokoloff *et al.*, 1985). In our hands this protein (which is unequivocally identified by the immune-replica technique) is found in both the detergent-rich phase and the aqueous phase, consistent with its dual location in granules.

Chromaffin-granule membrane proteins in the aqueous phase

Two-dimensional gels of the aqueous phase revealed three categories of protein: (1) contaminating matrix proteins; (2) unidentified hydrophilic proteins, possibly arising from the cytosol or from an extracellular location; and (3) several proteins known to be glycosylated, which are thought to be genuine membrane constituents.

The aqueous phase contains a residue (about 0.06%, w/v) of Triton X-114. It was therefore dialysed against Amberlite XAD-2 to remove as much detergent as possible. This resulted in precipitation of 20% of the protein. A two-dimensional electrophoretogram of this material (Fig. 3*a*) revealed several proteins, mostly glycoproteins (see below), previously characterized as membrane components (Gavine *et al.*, 1984). Proteins in categories (1) and (2) remained in solution (Fig. 3*b*) and are essentially the same as those removed from the membranes by Na_2CO_3 washing (Fig. 1*b*). Dialysis is thus a simple way of recovering membrane glycoproteins from this fraction.

Membrane glycoproteins

Because the membrane glycoproteins stain poorly with Coomassie Blue, separated proteins were transferred to nitrocellulose and decorated with three radioiodinated lectins (concanavalin A, wheat-germ agglutinin and lentil lectin) shown previously to bind to the major chro-

Table 2. Distribution of major chromaffin-granule membrane glycoproteins

The labelling of components, shown in Fig. 5, follows that of Huber *et al.* (1979) and Gavine *et al.* (1984); GpV has not been named previously. E, F and G are the components of dopamine β-hydroxylase, as revealed by antibody binding.

Designation		Phase		
Gavine *et al.* (1984)	Huber *et al.* (1979)	Phospholipid-rich	Detergent-rich	Pellet from dialysis of aqueous phase
B_1		+		+
B_2			+	
C			+	
	GpII		+	
D				+
E			+	
F	GpI		+	
G			+	
H		+		+
J		+		+
K		+		+
	(GpV)		+	
L		+		+
M				+
N				+
O				+
P	GpIV	+		
R	GpIII			+
T_1				+
T_2				+
U				+
W				+

maffin-granule components. Most of the glycoproteins in fact bind concanavalin A (Gavine *et al.*, 1984), and overlays of the three fractions with this lectin are shown in Fig. 4, together with a composite drawing in Fig. 5. In Fig. 4(*a*) we show an analysis of whole membranes, in order to show the relative intensity of concanavalin A binding of different components before they become concentrated in the separate phases. Figs 4(*b*) and 4(*d*) can be compared with Figs 2(*b*) and 3(*a*), which were stained for protein.

Fig. 4 demonstrates that two major categories of glycoprotein are found; some are revealed as a series of spots spread over a wide range of isoelectric points, whereas others are essentially homogeneous. In at least some cases the former category results from heterogeneous sialylation (Gavine *et al.*, 1984).

The three parts of Fig. 4 demonstrate again that there are clear differences between the three phases, few glycoproteins appearing in more than one (Table 2).

Application to other subcellular fractions

How applicable is the Triton X-114 method to other membranes? Our knowledge of the component proteins of adrenal-medullary organelles other than secretory granules is only fragmentary, but we have applied the method to a mitochondrial fraction, and to Golgi and rough-endoplasmic-reticulum fractions partially purified from microsomal fractions (Fig. 6). The Golgi-enriched fraction contains some plasma membrane; the enzymic markers for these fractions have been studied (J. G. Pryde & J. H. Phillips, unpublished work).

Fig. 6(*a*) demonstrates that, like chromaffin-granule membranes, the Golgi-enriched microsomal fractions yielded three phases with distinctive polypeptide compositions. In contrast, the less lipid-rich membranes from the mitochondrial and rough-endoplasmic-reticulum preparations (Figs. 6*b* and 6*c*) produced very little initial phospholipid-rich precipitate, although each yielded distinctive detergent and aqueous phases. The method therefore seems to be useful for the initial fractionation of proteins from a variety of types of membrane.

Distribution of ATPases

Chromaffin-granule membrane preparations are contaminated with traces of mitochondrial F_1-ATPase (Apps & Schatz, 1979; Apps *et al.*, 1983; Cidon *et al.*, 1983). The α-, β- and γ-subunits of this enzyme are revealed by immune replicas in Fig. 7(*a*): these components are found exclusively in the detergent-rich and aqueous fractions. This distribution is also found for the low-M_r dicyclohexylcarbodi-imide-reactive subunit of mitochondrial ATPase (which has an apparent M_r of about 17000; Fig. 7*b*). In contrast, the equivalent chromaffin-granule membrane component (Sutton & Apps, 1981), which is slightly smaller than its mitochondrial counterpart (Fig. 7*b*, track 1), is found solely in the phospholipid-rich phase, where other components of the granule's proton-translocating ATPase are also found (Percy *et al.*, 1985).

Other major dicyclohexylcarbodi-imide-reactive proteins of mitochondria can also be seen in Fig. 7(*b*). That of M_r 50000 is probably the β-subunit of F_1-ATPase (Satre *et al.*, 1982); the band of M_r about 34000 may be the mitochondrial phosphate transporter (Houstek *et al.*, 1981). Fig. 7(*b*), tracks 3, 4 and 5, may be compared with Fig. 6(*c*), in which an equivalent gel stained with Coomassie Blue is presented.

DISCUSSION

Separation of chromaffin-granule membrane proteins

The solubilization of chromaffin-granule membranes with Triton X-114, followed by temperature-induced phase separation (Bordier, 1981), has allowed us to separate proteins on the basis of their relative hydrophobicity. The addition of a final detergent-removal step, or the introduction of a preliminary Na_2CO_3-wash step, has allowed us to separate the most hydrophilic class, the adherent soluble proteins, and thus to remove proteins that are not true membrane constituents.

The initial precipitate is rich in hydrophobic proteins, typically the dicyclohexylcarbodi-imide-reactive protein and other constituents of ATPase I (Percy *et al.*, 1985). Warming the resultant solution leads to separation of another hydrophobic phase, characterized by its content of dopamine β-hydroxylase and cytochrome b_{561}. The final aqueous medium contains a mixture of soluble proteins and membrane glycoproteins, the latter precipitating on removal of residual detergent. After dialysis we found that the subunits of mitochondrial F_1-ATPase (identified by antibody overlays), and residual chromogranins, the major secretory proteins of the adrenal medulla, remained in solution. Dopamine β-hydroxylase (identified by antibodies and by lectins; Fig. 4*d*) is also found here, arising from its location in the matrix in addition to the membrane. In contrast, the major

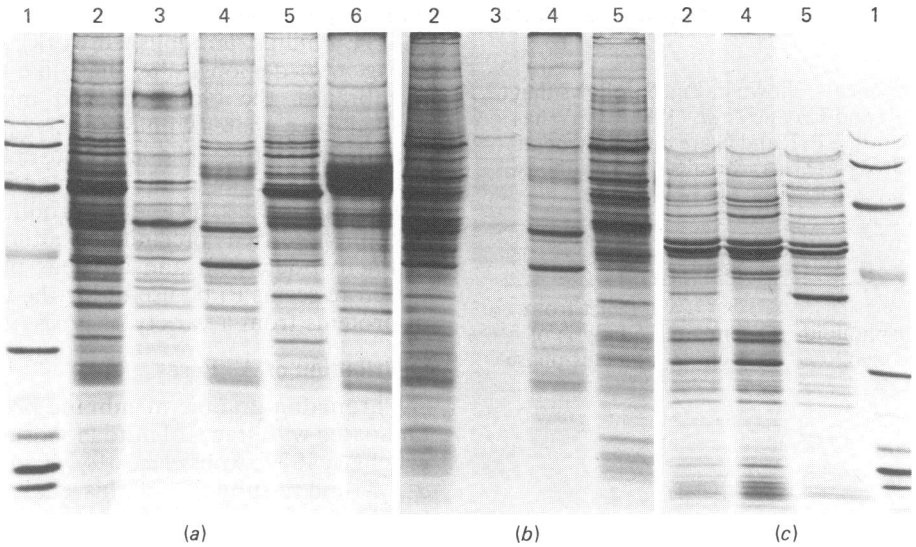

Fig. 6. Application of phase-separation method to other subcellular fractions

(*a*) Golgi-enriched microsomal fraction. (*b*) Rough-endoplasmic-reticulum-enriched microsomal fraction. (*c*) Mitochondria. Tracks: (1) standard proteins (see Fig. 1); (2) whole fraction; (3) phospholipid-rich phase (if formed); (4) detergent-rich phase; (5) aqueous phase; (6) chromaffin-granule membranes

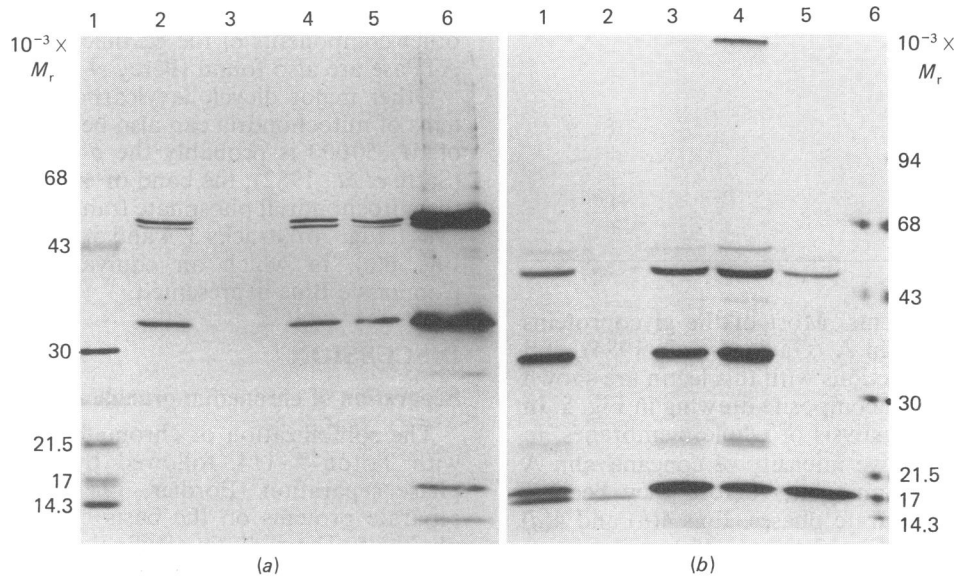

Fig. 7. Phase distribution of ATPase constituents

(*a*) Immune replica of electrophoretic separation of chromaffin-granule membrane proteins, obtained by using antiserum to mitochondrial F_1-ATPase. Tracks: (1) radioactive standard proteins (as Fig. 1); (2) whole chromaffin-granule membranes; (3) phospholipid-rich phase; (4) detergent-rich phase; (5) aqueous phase; (6) purified mitochondrial F_1-ATPase. (*b*) Autoradiograph of one-dimensional separation of proteins labelled by *NN'*-dicyclohexyl[^{14}C]carbodi-imide. Tracks: (1) mixture of phospholipid-rich phase from chromaffin-granule membranes with detergent-rich phase from mitochondria; (2) whole chromaffin-granule membranes; (3) whole mitochondrial membranes; (4) detergent-rich phase from mitochondria; (5) aqueous phase from mitochondria; (6) standard proteins.

glycoproteins H, J, K and R (nomenclature of Gavine *et al.*, 1984) were precipitated.

These results therefore do not support the idea that chromogranin A, or any portion of it, may be an integral membrane protein (Settleman *et al.*, 1985). None is revealed by Coomassie Blue staining of the precipitated proteins. The glycoproteins that precipitate are presumably the most hydrophilic of the integral membrane components of the chromaffin granule; many were found to be present to some extent in the phospholipid-rich precipitate as well. They are clearly intrinsic membrane components, even though they do not partition into the detergent phase, as originally suggested by Bordier (1981). A similar partitioning of acetylcholine-receptor pentamers into the aqueous phase has been reported by Maher & Singer (1985).

Chromaffin-granule membrane glycoproteins

We have previously reported characterizations of the membrane and soluble glycoproteins of chromaffin granules (Gavine *et al.*, 1984; Apps *et al.*, 1985). Much of the complexity of the former analysis has been resolved by the present separation of proteins into three distinct fractions, and the two-dimensional electrophoretograms enable us to observe those components that are highly heterogeneous. Results are summarized in Fig. 5 and Table 2. We have identified the glycoproteins by using the nomenclatures of both Gavine *et al.* (1984) and Huber *et al.* (1979): this is done by comparing binding of the different lectins, as well as by the shapes and positions of spots on the gels.

Early analyses of these glycoproteins concentrated on the major components dopamine β-hydroxylase, glycoprotein II and glycoprotein III (Fischer-Colbrie *et al.*, 1982). Glycoprotein II appears to be highly heterogeneous (M_r 84000–100000; we have indicated this by the letters a and b in Fig. 5); the wide range of pI presumably results at least in part from incomplete sialylation, since the molecule contains on average 17 μmol of sialic acid/100 mg of protein (Fischer-Colbrie *et al.*, 1982).

Both glycoprotein II and glycoprotein III have a high affinity for wheat-germ agglutinin: the low affinity of the latter for concanavalin A makes it hard to detect in Fig. 4. Neuraminidase treatment fails to remove all of its heteogeneity, suggesting the possibility of other forms of modification (Gavine *et al.*, 1984).

We believe that glycoprotein IV is identical with the constituent of that name described by Huber *et al.* (1979). A constituent with M_r about 47000, it binds concanavalin A well, and is the most characteristic glycoprotein of the phospholipid-rich phase. It appears to be different from another minor constituent, equally heterogeneous, but mainly found in the detergent-rich phase, that we have called glycoprotein V.

The glycoproteins are summarized in Table 2. We have omitted all glycoproteins that remained in solution after dialysis of the aqueous phase (see Fig. 5), on the assumption that these are either matrix components or extracellular contaminants of the granule preparation. This assumption may not, of course, be valid in every case (Kuchel *et al.*, 1978; Maher & Singer, 1985).

Generality of the method

The method that we describe in this work appears to be generally useful. Triton X-114 is an effective detergent for solubilizing membrane components, and the simple manipulations of temperature and centrifugation permit an easy first stage of separation of membrane proteins. It is clear that different membranes will behave in characteristic ways. Bordier (1981), for example, found only the detergent-rich and aqueous phases when human erythrocyte membranes or whole myeloma cells were treated, and a similar result was found with platelets (Clemetson *et al.*, 1984) and granulocytes (Pember *et al.*, 1984). We find, however, that relatively lipid-rich membranes yield an additional phase which is insoluble at 0 °C, and which contains its own characteristic family of hydrophobic proteins (Figs. 1 and 6). Furthermore, the two treatments described (Na_2CO_3 washing, or dialysis of the aqueous phase) permit a further separation of truly soluble proteins from the most hydrophilic of the intrinsic membrane glycoproteins.

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