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ORGANISATION OF THE PROTEINS OF THE CHROMAFFIN GRANULE MEMBRANE

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Summary

The organisation of the protein components of bovine chromaffin granules has been investigated by labelling or digesting intact granules or broken membranes with the following reagents: lactoperoxidase/$Na^{125}I$ as a reagent for tyrosine residues, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid as a reagent for cysteine residues, pronase, and galactose oxidase/KB^3H_4. Following treatment, membranes were purified and washed and proteins were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Rather more than 60 bands were resolved, of which about 40 were relatively intense and reproducible. The bands were classified according to their molecular weights and sensitivity to reagents. Penetration of the membranes by the reagents was assessed by examination of intragranular proteins.

The majority of chromaffin granule membrane polypeptides became labelled when intact granules were treated with impermeant reagents. Eleven were probably protected in the intact granules, reactive sites becoming exposed only on membrane lysis. By contrast, carbohydrate moieties of glycoproteins appear to be exposed only on the matrix side of the membrane. Two proteins were shown to span the membrane, although this is probably an underestimate.

Introduction

Rather little is known at present about the assembly and organisation of secretory granule membranes. Studies on protein topography have concen-

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; 1,5-AEDANS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid.

trated on the plasma membrane; work on intracellular membranes has so far largely been limited to energy-transducing membranes and endoplasmic reticulum (reviewed by DePierre and Ernster [1]). The purity of secretory granule preparations, and the simplicity of granule organisation, suggest that the study of such organelles should be a useful objective. Such studies may eventually aid the identification of proteins involved in phenomena such as exocytosis and organelle transport within the cell, as well as the organisation of the granule contents.

Chromaffin granules are the secretory granules of the adrenal medulla, specialised for the storage and release of catecholamines. What is known of their biogenesis and biological function has recently been reviewed [2]. They are seen in electron micrographs as opaque membrane-bound granules with a diameter of about 200 nm. Because they contain a high concentration of protein they are easily isolated from other subcellular components by density gradient centrifugation; they are, however, readily lysed by hypotonic solutions, after which their membranes can be separated from the released matrix components.

The proteins of chromaffin granule membranes are at present poorly characterised. The following enzyme activities have been identified as components of the membrane [3]: dopamine β-hydroxylase (EC 1.14.17.1); proton-translocating Mg^{2+}-ATPase (EC 3.6.1.3); NADH:(acceptor) oxidoreductase (EC 1.6.99.3) and phosphatidylinositol kinase (EC 2.7.1.67). A number of other activities are disputed. In addition, the membrane contains cytochrome *b*-561 [4] and α-actinin [5]. It may be presumed to contain, additionally, permeases for intragranular components such as catecholamines, nucleotides, calcium and ascorbate.

Several attempts have been made recently to investigate the topography of components within the chromaffin granule membrane. An extensive survey of the lipids, based on enzymic degradation and chemical labelling, failed to reveal any marked asymmetry of distribution [6]. Among the enzymes, dopamine β-hydroxylase is unusual in that it is located both within the granule matrix and in the membrane: the latter has been shown to be primarily located on the matrix side of the membrane in a study using radioactive diazobenzene sulphonate and microcomplement fixation [7], and this is consistent with the known latency of its catalytic activity [8]. A number of glycoproteins have been demonstrated by electrophoretic analysis of membranes in sodium dodecyl sulphate [9—11]; galactose oxidase digestion followed by radioactive borohydride labelling suggests that their carbohydrate moieties are on the matrix side of the membrane [10], a result consistent with electron microscopic studies of concanavalin A binding sites [9,10].

In the work reported in this paper we have separated membrane proteins of bovine chromaffin granules by electrophoresis in polyacrylamide slab gels in the presence of sodium dodecyl sulphate, and have attempted to localise their position by using a variety of impermeant enzymic (lactoperoxidase, pronase, galactose oxidase) and one non-enzymic (1,5-AEDANS) probes. Our data show that most proteins are accessible on the cytoplasmic surface of the granules, although we confirm that the carbohydrate portions of glycoproteins face the matrix.

Materials and Methods

Solutions were generally buffered with 10 mM Hepes, pH 7.0 (Sigma London Chemical Co. Ltd., Poole, Dorset, U.K.). The pH of 1.0 M Hepes was adjusted with NaOH. Pronase is a nonspecific protease preparation from *Streptomyces griseus* (Sigma type VI). Galactose oxidase was Sigma type IV, from *Dactylium dendroides* (85 units/mg protein). Lactoperoxidase and concanavalin A were obtained from the Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K. Cholesterol oxidase was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Butylated hydroxytoluene was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K. *N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid (1,5-AEDANS) was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. $Na^{125}I$ was used carrier free. KB^3H_4 was 332 Ci/mol.

Chromaffin granule membranes. Crude chromaffin granules were prepared as a particulate fraction from bovine adrenal medullae by homogenisation of the tissue in 0.3 M sucrose buffered with 10 mM Hepes, pH 7.0, centrifugation for 10 min at 500 × *g* at 4°C, followed by centrifugation of the resultant supernatant for 20 min at 17 000 × *g* at 4°C. The pellet was washed twice with removal of the bulk of the mitochondria by resuspension and centrifugation [12]. Following treatment with reagents as detailed below, the entire suspension (about 1 ml) was layered over 5 ml of 1.8 M sucrose buffered with 10 mM Hepes, pH 7.0 (alternatively, 2—3 ml of suspension was layered over 20 ml); centrifugation was for 1 h at 120 000 × *g* at 4°C. The supernatant was discarded, the inner surface of the centrifuge tube was cleaned carefully, and the granules were then lysed by suspension in 9 ml of 10 mM Hepes, pH 7.0. The suspension was centrifuged for 1 h at 160 000 × *g* at 4°C: the supernatant was saved for analysis of matrix proteins.

Membranes were freed from contaminating traces of matrix proteins in the pellet by subjecting them to two cycles of osmotic shock. The pellet was suspended in 0.25 ml of 1.8 M buffered sucrose, and then diluted to 10 ml with 10 mM Hepes. This procedure was repeated after centrifugation. The membranes were finally suspended in 10 mM Hepes to a concentration of 5—10 mg/ml.

Treatment with reagents. Granules were iodinated following suspension in 0.5 ml 0.3 M buffered sucrose (15—20 mg/ml) to which was added 0.8 unit of lactoperoxidase, 200 μCi $Na^{125}I$ and 2 μg butylated hydroxytoluene [13] in ethanol (final ethanol concentration was 0.2%). To this mixture was added 3 μmol H_2O_2 (2-μl portions containing 0.2 μmol were added every 4 min over 1 h at 12—15°C). Reaction was terminated by addition of β-mercaptoethanol to 30 mM. The granules were purified, lysed and the membranes washed in solutions containing 10 mM KI. Membranes were iodinated by a similar method, except that the enzyme concentration was reduced ten-fold.

Sulphydryl groups were labelled with 1 mM or 3 mM 1,5-AEDANS. Reaction was carried out in the dark either at room temperature for 15 or 30 min or at 4°C overnight. Reaction was terminated by addition of excess β-mercaptoethanol. Pretreatment of membranes or granules with 1 mM cysteine at 0°C

prior to labelling made no difference to the labelling pattern.

Pronase digestion was usually performed using 0.1 mg of pronase/ml at room temperature. When the time of digestion varied, incubations were arranged to end simultaneously, but all samples were kept at room temperature for an identical time; controls lacked enzyme. Incubations were terminated by dilution with ice-cold 10 mM Hepes or 0.3 M buffered sucrose.

For labelling of carbohydrate residues granules or membranes were incubated for 3 h at 20°C with 34 units/ml of galactose oxidase [14]. After two washes by centrifugation, approximately 250 μCi KB^3H_4 were added, and reaction was continued for 30 min at 20°C. This was followed by addition of excess $NaBH_4$.

Polyacrylamide gel electrophoresis. Membrane and matrix proteins were separated by electrophoresis in the presence of sodium dodecyl sulphate in polyacrylamide slab gels, basically following the method of Laemmli [15]. The gels were generally composed of 8% acrylamide, or of linear gradients of 8—20% acrylamide, and were always overlaid with stacking gels of 3% acrylamide. Standards were always run, as shown in Fig. 1. Each sample slot contained approximately 100 μg of protein (200 μg when stained for glycoproteins).

Samples were solubilised by heating for 4 min at 100°C in a mixture containing 60 mM Tris-HCl, pH 6.8, 10% glycerol, 0.001% bromophenol blue, 5% sodium dodecyl sulphate and 40 mM dithiothreitol (the latter component was omitted when gels were run of non-reduced proteins). In the case of membranes that had been exposed to pronase, the Tris buffer was replaced by 50 mM potassium biphthalate, pH 4.0 [16]: in this case 1 vol. of sample at ice temperature was added to 3 vols. of dissociation buffer at 100°C and the mixture heated at 100°C for 3 min.

Gels (16 cm × 16 cm) were normally run at room temperature at about 40 mA. They were then stained with Coomassie brilliant blue [15] or by periodic acid-Schiff reagent [17]. Fixation of gels in 12.5% trichloroacetic acid before staining was found to have no effect on the staining pattern, and was omitted. After photography individual lanes were excised and scanned in a Gilford gel scanner (model 2520). Fluorescent gels were scanned before staining: for this purpose the gel scanner was modified essentially as described by Ragland et al. [18]. After scanning, the gels were stained for protein. Fluorescent lectin binding to proteins in gels [19] was performed as described by Cahill and Morris [11].

Radioactive gels were cut into 1 mm slices using an array of razor blades. Gels containing ^{125}I were counted directly in a γ-counter; gel slices containing ^3H were solubilised and counted by the method of Zaitlin and Harikarusubramania [20].

Biochemical assays. Protein was assayed by the method of Bradford [21]. Cholesterol assays were performed using cholesterol oxidase. Mg^{2+}-ATPase was assayed by incubating membranes in 1 mM MgATP, 10 mM Hepes, pH 7.0, for 30 min at 37°C. Phosphate was determined according to Muszbek et al. [22]. Phosphatidylinositol kinase and dopamine β-hydroxylase were assayed as described previously [23], except that tyramine was present at 0.2 mM in the latter assay. NADH:ferricyanide oxidoreductase and the reduced vs. oxidised spectra of membranes were determined as described by Flatmark et al. [4].

Results

Electrophoresis of purified chromaffin granule membranes

Chromaffin granules are obtained in a high state of purity by centrifuging them through 1.8 M sucrose. After this density gradient procedure their contamination by other organelles is negligible [24,25]. However, they are rendered rather fragile by this treatment. In experiments in which granules were labelled or digested, we therefore treated crude granules (contaminated with mitochondria), and then followed this by density gradient centrifugation, lysis and membrane preparation. In this way intact granules are separated from membranes arising by lysis during labelling. Broken membrane preparations were made by lysing purified granules, followed by two cycles of osmotic shocking. In all cases matrix proteins released by lysis were saved, so that penetration of intact granules by the labelling reagent could be assessed by examining gels of these proteins.

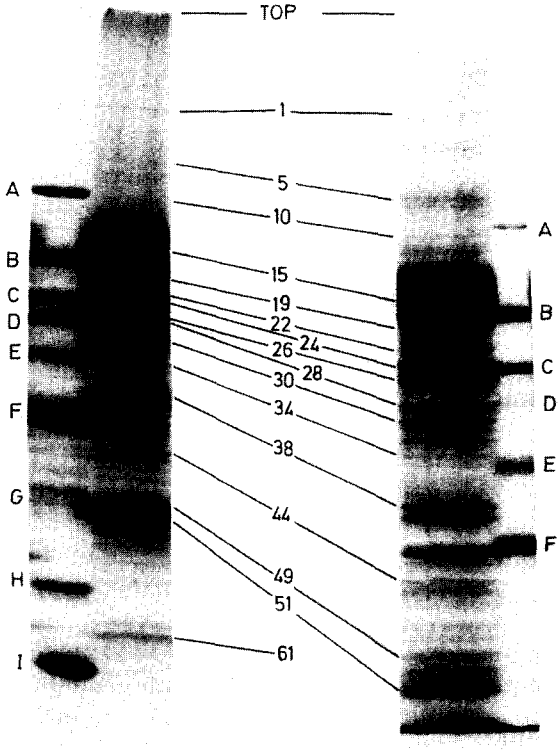

Fig. 1. Proteins of chromaffin granule membranes separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Membranes were solubilised and their proteins (visualised here by Coomassie blue staining) were separated by electrophoresis in (left) an 8—20% polyacrylamide slab gel and (right) an 8% gel. Band numbers used throughout this paper are given. Dopamine β-hydroxylase is found at bands 14 and 15, Mg^{2+}-ATPase at bands 28, 29 and 44 and chromomembrin B at band 51. Standard polypeptides with molecular weights [26] are phosphorylase *a* (A: 94 000), bovine serum albumin (B: 68 000), catalase (C: 60 000), γ-globulin H chain (D: 50 000) ovalbumin (E: 43 000), glyceraldehyde phosphate dehydrogenase (F: 36 000), γ-globulin L chain (G: 23 500), soya bean trypsin inhibitor (H: 22 000) and cytochrome *c* (I: 11 700).

Although we attempted to perform all electrophoretic separations under closely similar conditions, the resolution of the gels was variable. Main features of band patterns were always recognisable, and minor bands were identified as far as possible. Because of this variability, the results we present are based on several experiments with each reagent. In all cases gels containing labelled material were stained for protein, and were scanned at least twice. Careful correction was needed for artefacts arising from swelling or shrinking of the gels, and alignment of the gels in the scanning apparatus.

It can be seen from Fig. 1 that at least sixty bands can be resolved on single-dimension gels under optimal conditions, although many of these are minor components. Some bands clearly contain more than one polypeptide, since partial resolution sometimes occurs. This complicates the interpretation of labelling experiments. In Fig. 1, however, we have aligned major bands in the two gel systems that we have used, and indicate the band numbering system that we use throughout this paper. Band patterns are summarised in Table I.

Inevitably, the membranes are contaminated to some extent by adsorbed proteins and other attached membranes such as plasma membranes [27,28].

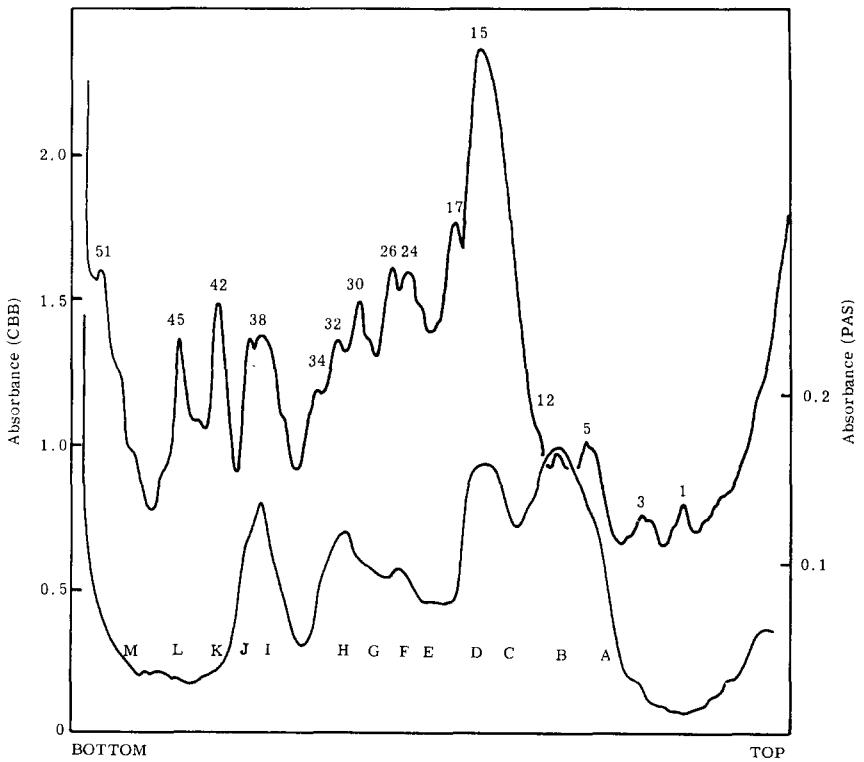

Fig. 2. Chromaffin granule membrane glycoproteins. Purified chromaffin granule membranes were subjected to electrophoresis (8% gel). The gel was stained by the periodic acid-Schiff method (PAS) and scanned (lower trace). After several days it was further stained with Coomassie brilliant blue (CBB) and rescanned (upper trace). The gel is overloaded with protein in order to increase the PAS staining for scanning: resolution of the protein stain is thus not optimal, but major band numbers have been indicated. Regions of the glycoprotein trace have been labelled with letters, as indicated in the text. Regions K—M do not stain with PAS reagent, but can be visualised with fluorescent concanavalin A.

Minor bands in the gels may arise from these, and in our discussion of labelling patterns we therefore concentrate on the major components, omitting about 20 faint bands from detailed consideration.

We also stained gels for carbohydrate by using the periodic acid-Schiff staining method [17] (Fig. 2). This method is not very sensitive and consequently the gels have to be overloaded in terms of their protein content: this leads to a loss of resolution of the Coomassie blue-stained bands, which, coupled with the broadness of glycoprotein bands in general, makes it difficult to identify periodic acid-Schiff-staining regions with particular polypeptides. Major glycoprotein regions have been identified by letters (A—M) in Fig. 2. These are not all resolved as separate peaks, nor are all the regions visualised by periodic acid-Schiff staining; they are identified on the basis of fluorescent lectin binding (Ref. 11, and our own work) and dansyl hydrazine fluorescence [29] as well as staining properties, and are summarised in Table II. The profiles we obtained were not dissimilar to those of other workers [9—11,30].

Major proteins of chromaffin granule membranes

The gel patterns are dominated by a broad, dark-staining region with an apparent molecular weight just above 70 000. The enzyme dopamine β-hydroxylase migrates here. It can be identified by two criteria: it is a glycoprotein, not only staining well in gels by the periodic acid-Schiff method, but also binding lectins such as concanavalin A [10,11]; second, it characteristically migrates as a dimer of molecular weight 150 000 when subjected to electrophoresis in the presence of sodium dodecyl sulphate, but in the absence of reducing agent [31,32]. Also running in this region of the gel is the major matrix protein, chromogranin A (this is shown in Fig. 8). This is probably a glycoprotein, staining with periodic acid-Schiff reagent and dansyl hydrazine, but not binding concanavalin A. Several broad bands found in this region of the gel are rather poorly resolved in our hands. Running gels in the absence of reducing agent removes the major bands 14 and 15 (and the lectin-binding properties of this region), although a number of minor bands remain. However, we performed all our labelling analyses on gels run with reduced material: this not only avoids problems due to disulphide linking of polypeptides, but we found that much protein material (about 30%) remains at the top of the gel when reducing agent is absent.

Proteins present in the granule lysate can be seen in Fig. 8. The dominant chromogranin bands can be seen running ahead of the dopamine β-hydroxylase region. In order to avoid contamination, we attempted to remove chromogranin A from our preparations by subjecting the membranes to three cycles of osmotic shock. Inspection of gels of membrane proteins that had not been reduced suggested that, if present at all, chromogranin A was a very minor component compared with dopamine β-hydroxylase. However, band 13 is an ill-defined staining region, and we cannot eliminate the possibility that it results from some chromogranin overlapping with bands 14 and 15 (although comparison of mobilities on Fig. 8 seems to make this unlikely).

The proton-translocating ATPase has three prominent subunits (α, β and γ) that can be purified from chromaffin granule membranes by solvent extraction [25]. We have partially purified this enzyme and showed that it can be identi-

fied with bands 28, 29 and 44 of our gels. Actin is a very minor component of the membranes [5,33] and is tentatively identified on the basis of its molecular weight as band 35. A different protein of similar molecular weight known as synaptin [34] probably also migrates in this region.

A major band with molecular weight approximately 22 000 (easily seen in the gradient gel, Fig. 1) has been named chromomembrin B [35]. Bartlett and Smith [36] point out that this band appears to be heterogeneous, resolving into a doublet at high acrylamide concentrations. In our hands this region is often resolved into three bands (49—51). König et al. [7] reported that chromomembrin B was located on the cytoplasmic surface of the granule membrane.

We are currently unable to identify any further bands.

Lactoperoxidase-catalysed iodination

In preliminary membrane-labelling experiments we used reagents for amino groups. This was found to be unsatisfactory because of the large incorporation into phospholipids and catecholamines, giving high backgrounds on the gels. Use of lactoperoxidase-catalysed iodination [37] had a similar disadvantage: when labelling intact granules much radioactivity was incorporated into leaking catecholamines, greatly diminishing the incorporation into protein. We there-

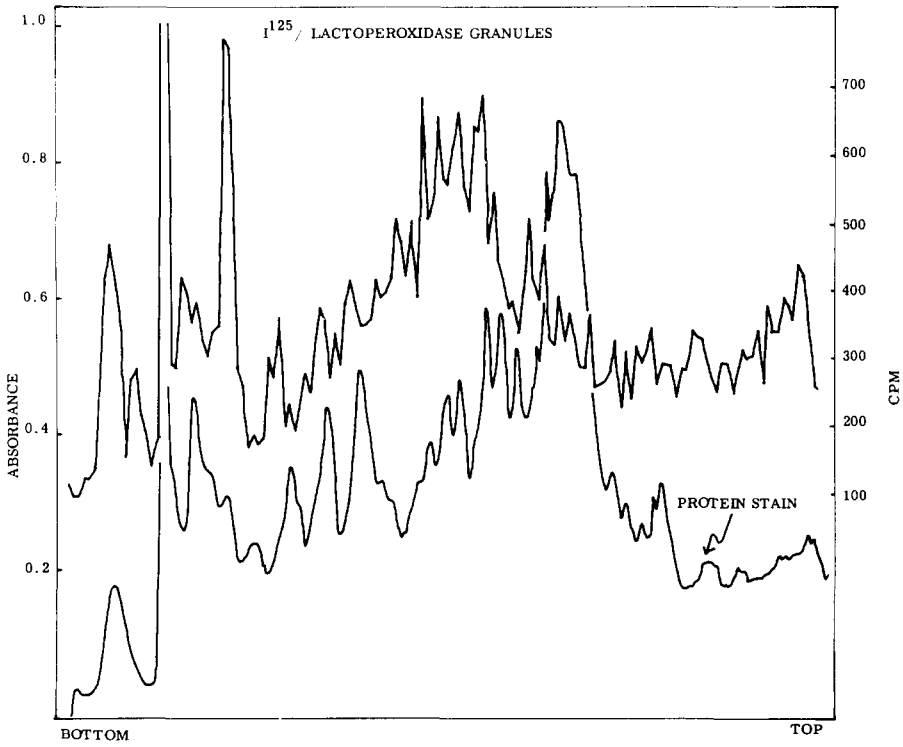

Fig. 3. Iodination of intact chromaffin granules by lactoperoxidase. Intact chromaffin granules were labelled with ^{125}I in the presence of lactoperoxidase. The granules were then purified and lysed, and their washed membranes were then subjected to electrophoresis (8% gel); following Coomassie blue staining and scanning, the gels were sliced and radioactivity determined. The tracker dye front is found in the sharp peak just above the bottom of the gel.

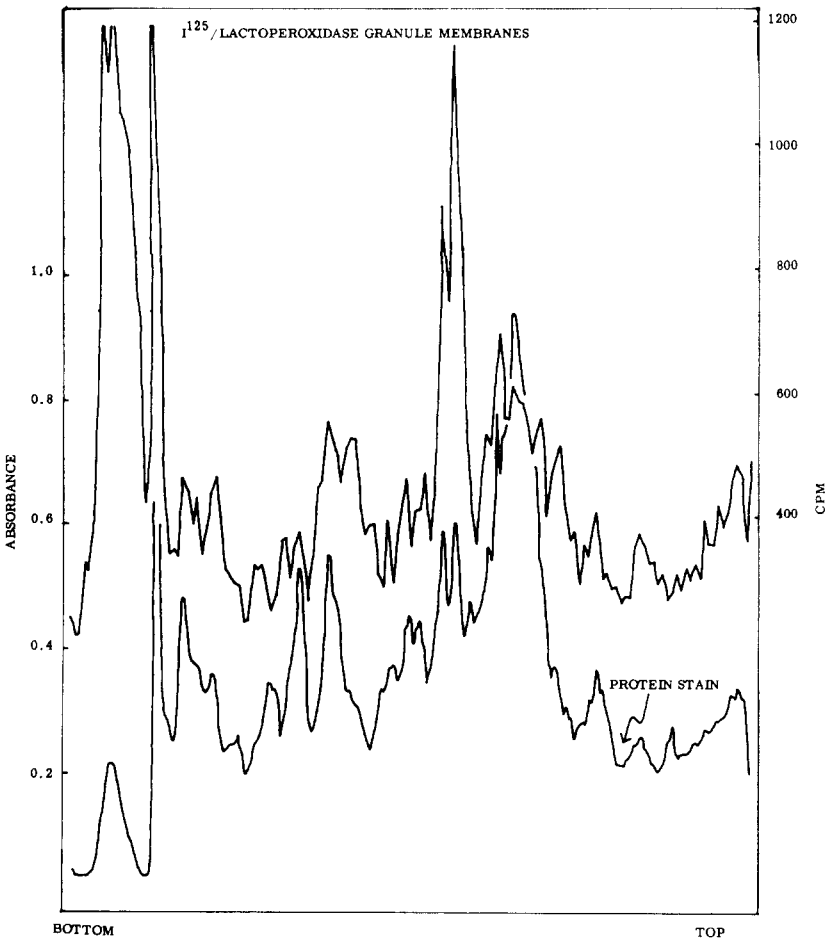

Fig. 4. Iodination of broken chromaffin granule membranes by lactoperoxidase. Purified chromaffin granule membranes were labelled with ^{125}I in the presence of lactoperoxidase. After washing they were subjected to electrophoresis as in Fig. 3.

fore found autoradiography rather unsatisfactory, and preferred instead to assay the radioactivity in sliced gels.

In Figs. 3 and 4 we present the results of experiments in which intact granules and broken membranes were iodinated. The most striking features of these experiments are the following: (1) Many small radioactive bands appear. It is hard to assess these when they are confined to a single gel slice, since this could be caused by unequal amounts of gel being recovered from different slices. In general such fluctuations were discounted unless they were very large or occurred in two consecutive gel slices. (2) A high radioactive background is seen in the central region of one of the gels, easily visualised as a grey area in autoradiographs. We have no explanation for this, as it does not occur systematically with membranes labelled either in the broken or intact state. (3) Discounting this uneven background, certain components are clearly more

heavily labelled in the membrane state than in the intact granules (e.g. bands 24 and 38). By contrast, one band (49) is always much more heavily labelled in intact granules. (4) The dopamine β-hydroxylase region of the gel is relatively poorly labelled. While some bands in this region are labelled in intact granules, more label is incorporated here in broken membranes. This can be clearly demonstrated by running gels in the absence of reducing agent, when dopamine β-hydroxylase is well separated from other components.

We demonstrated that iodination failed to take place in the absence of lactoperoxidase, and we also showed that proteins in the matrix were completely unlabelled following treatment of intact granules. We observed, however, that high concentrations of catecholamines were inhibitory for protein iodination, so that labelling of chromogranins might not be expected even if a reactive species of iodine did penetrate the membrane; by the same token, labelling of proteins exposed at the inner surface of the membrane would presumably also be suppressed.

An analysis of lactoperoxidase-labelling experiments is presented in Table I. This is derived from four separate experiments (of which Figs. 3 and 4 represent one) with different preparations of granules and membranes. In the table we indicate major radioactive bands, and also Coomassie blue-stained bands that were not clearly resolved in the radioactive profiles.

Reaction with 1,5-AEDANS

N-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid (1,5-AEDANS) is a fluorescent sulphydryl reagent [38]. We treated intact granules and broken membranes with it, separated the membrane proteins by electrophoresis and scanned the gels for fluorescence. The same gels were then stained for protein and scanned again. The results of a typical experiment are shown in Fig. 5.

Although small differences can be seen between the two fluorescent profiles, very few of these were shown to be completely reproducible: the labelling pattern was very similar when membranes were labelled in intact granules or in the broken state. A few protein bands were very heavily labelled, notably the high molecular weight doublet (bands 1 and 2) and a component running just ahead of dopamine β-hydroxylase (bands 16 and 17). This enzyme itself (bands 14 and 15) was essentially unlabelled in either membrane state, as shown by running gels of solubilised membranes in the absence of reducing agent. In general, the resolution obtained by scanning fluorescent gels was not as good as that obtained by photographing gels stained with Coomassie brilliant blue.

In order to check whether the reagent was penetrating the membrane when intact granules were used, we compared the labelling of the matrix proteins isolated from such granules with matrix proteins labelled when highly purified granules had been broken by freeze-thawing in the presence of the reagent. The fluorescence of equivalent amounts of protein is shown in Fig. 6; there was very little labelling in the former case, suggesting that 1,5-AEDANS is indeed inpenetrant under our labelling conditions (16 h at 4°C). We therefore conclude that most reactive groups for this reagent are located on the cytoplasmic surface of the granule; with a few exceptions (discussed below), any that are revealed by breaking the membrane are not sufficiently resolved on the gels to be detected by our procedure.

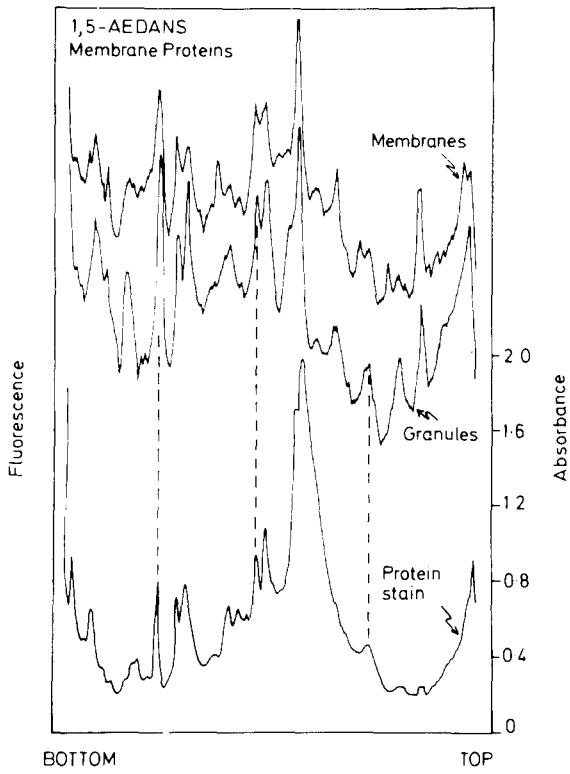

Fig. 5. Labelling of chromaffin granule membranes with 1,5-AEDANS. Intact chromaffin granules or broken membranes were labelled with 1,5-AEDANS. Purified washed membranes were then subjected to electrophoresis (8% gel). After scanning for fluorescence the gels were stained for protein with Coomassie brilliant blue (lower trace). Middle trace: fluorescence of gel of proteins from labelled intact granules. Upper trace: fluorescence of gel of proteins from broken membranes.

Reaction with pronase

Bender et al. [16] used pronase digestion to investigate the distribution of erythrocyte membrane proteins. We investigated the effect of pronase digestion on a number of activities of chromaffin granules (Fig. 7): these activities are given in terms of the cholesterol content of the membranes recovered after digestion, in view of the diminishing protein content. Following digestion, the granules are purified by density gradient centrifugation; in other words, only intact granules are analysed. About 30% of the membrane protein can be removed from granules by proteolysis without the granules disintegrating (under these conditions of digestion cholesterol recovery indicates that only 30% of the granules are lost by lysis). When broken membranes are digested, 70% of the protein is lost within 10 min: the remaining protein is pronase resistant.

As expected, ATPase, phosphatidylinositol kinase and NADH oxidation activities are lost when intact granules are treated with pronase. Dopamine β-hydroxylase is resistant except when broken membranes are subjected to digestion. In Fig. 7B its activity is compared with that of membranes kept at

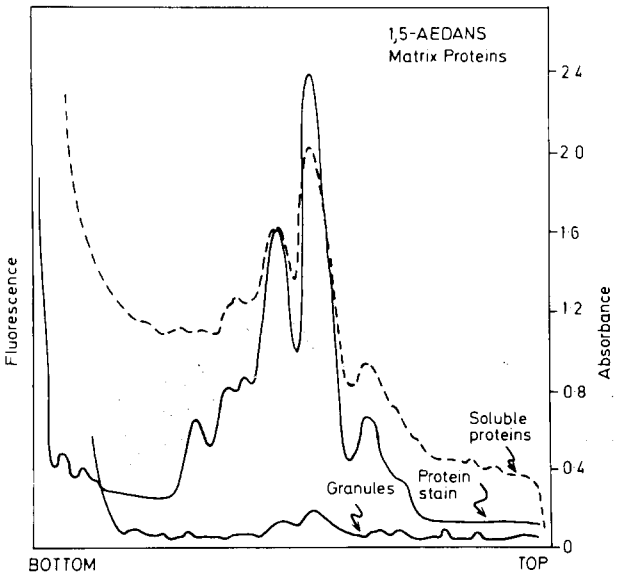

Fig. 6. Labelling of chromaffin granule matrix proteins with 1,5-AEDANS. Intact chromaffin granules were labelled with 1,5-AEDANS as in Fig. 5. Following purification, matrix proteins were collected from the granule lysate and subjected to electrophoresis (8% gel). The gel was scanned for fluorescence (lower curve; ———), then stained for protein and rescanned (middle curve; ———). Another portion of intact chromaffin granules was purified by density gradient centrifugation, lysed by subjection to freezing and thawing and then the broken granules were treated with 1,5-AEDANS under the same conditions as the intact granules above. Matrix proteins were then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. The fluorescence of the resulting gel (- - - - -) was then normalised by comparing the protein absorbance values of the two gels.

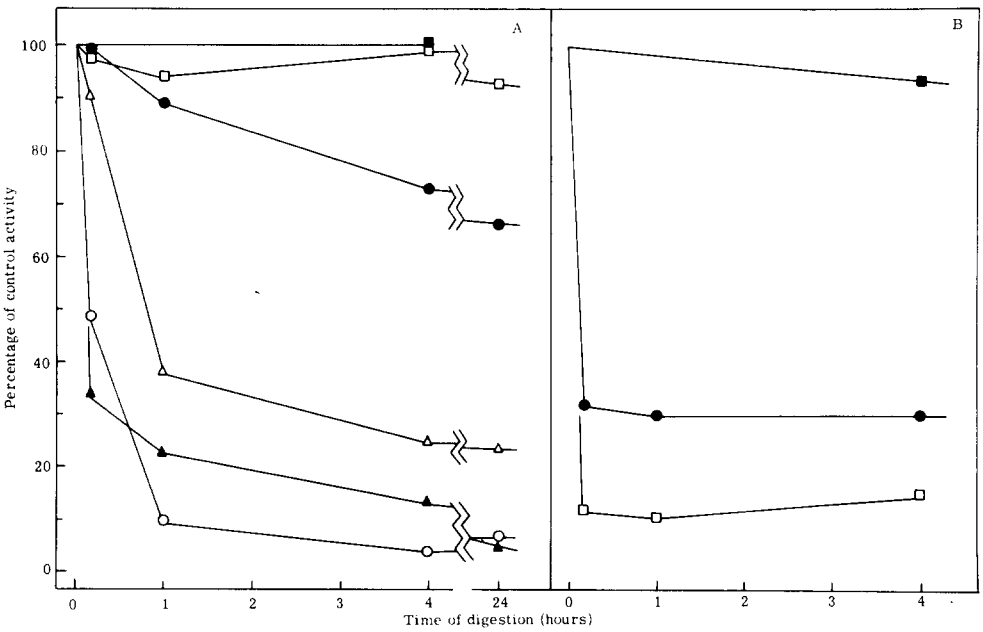

Fig. 7. Digestion of granules and membranes by pronase. Intact granules (A) or broken membranes (B) were subjected to digestion with pronase (0.1 mg/ml) at room temperature for the times shown. Measurements were made on purified membranes. Control activities of membranes which had not been incubated with pronase are given in brackets : protein content (●, 1.06 mg/mg cholesterol); dopamine β-hydroxylase (□, 12.5 nmol/mg cholesterol per min); NADH:ferricyanide oxidoreductase (△, 110 nmol/mg cholesterol per min); phosphatidylinositol kinase (▲, 0.50 nmol/mg cholesterol per min); cytochrome *b*-561 (■, 0.11 ΔA_{561} absorbance unit/mg cholesterol); Mg^{2+}-ATPase (○, 52.9 nmol/mg cholesterol per min).

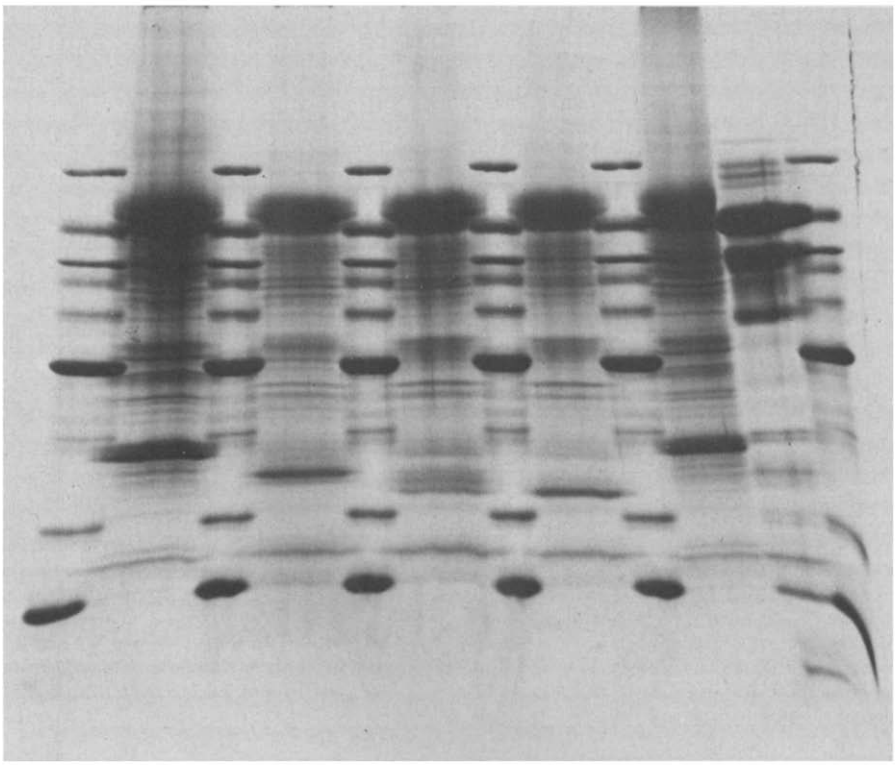

Fig. 8. Pronase treatment of intact chromaffin granules. Intact chromaffin granules were treated with pronase (0.1 mg/ml) at room temperature. After treatment purified membranes were isolated and subjected to electrophoresis (8—20% gradient gel). From the left, lanes 1, 3, 5, 7, 9 and 12 contain standard proteins, as in Fig. 1. Lanes 2 and 10 contain membranes from granules kept on ice or at room temperature for 4 h. Lanes 4, 6 and 8 contain membranes from granules incubated with pronase for 10 min, 1 h and 4 h, respectively. Lane 12 contains a sample of matrix proteins.

0°C. If broken membranes are warmed to room temperature for 4 h in the absence of pronase, nearly 50% of activity is lost, presumably due to endogenous protease, against which the enzyme is protected in intact granules. Some activity (about 10%) appears to resist proteolysis in the presence of pronase, possibly because the enzyme is protected by membrane vesiculation. The cytochrome *b*-561 chromophore is not lost from the membrane.

Examination of gels shows that many polypeptides are degraded by pronase (Fig. 8), although rates of degradation vary greatly. In the case of intact granules, the dopamine β-hydroxylase region retains its characteristic prominence in the profile. A detailed analysis of band susceptibility is presented in Table I, although in a few places analysis is complicated by the appearance of new bands. The major protein component of chromomembrin B is of interest. It appears to rapidly lose a fragment of approximately 1500 daltons, followed more slowly by another fragment of similar size. We conclude this from the very high intensity of staining of the newly produced bands, which seem unlikely to arise from any other single component of the mem-

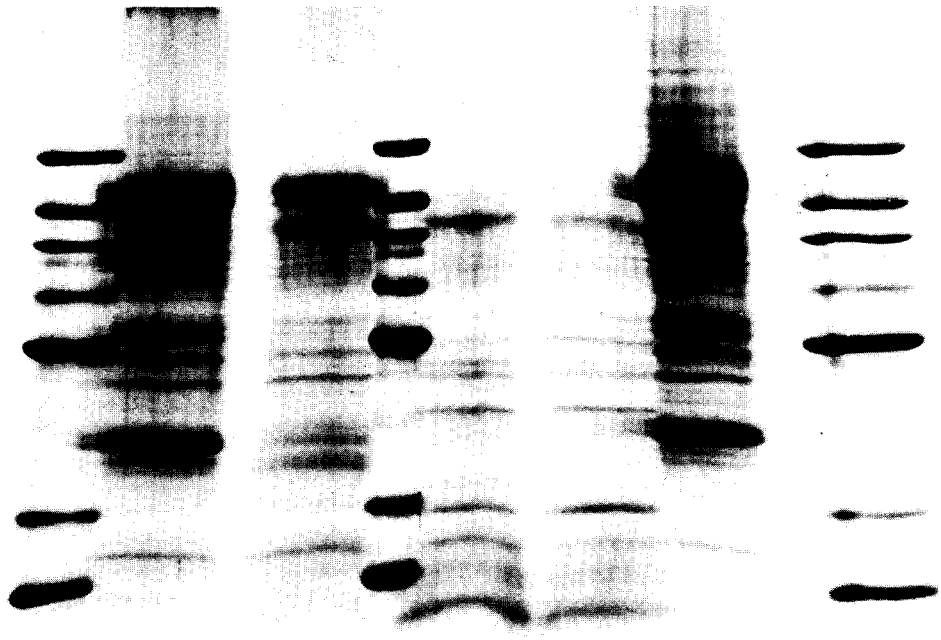

Fig. 9. Pronase treatment of broken chromaffin granule membranes. Broken chromaffin granule membranes were treated with pronase for 30 min at room temperature. After washing they were subjected to electrophoresis (8—20% gradient gel). From the left, lanes 1, 4 and 8 contain standard proteins, as in Fig. 1. Lanes 2 and 7 contain untreated membranes. Lanes 3, 5 and 6 contain membranes treated with pronase at 0.02 mg/ml, 0.2 mg/ml and 0.5 mg/ml, respectively.

brane. By contrast (Fig. 9), when broken mebranes are digested, the chromomembrin B is rapidly and completely lost by proteolysis.

In Fig. 9 we show the result of an experiment in which broken membranes were digested for 1 h with different concentrations of pronase. It can be seen that a few bands are totally resistant to pronase digestion, and, as in Fig. 8, some digestion products appear as new bands on the gel. These presumably arise from polypeptide products embedded in the membrane, which remain associated with the membrane during purification.

The dopamine β-hydroxylase polypeptides become susceptible to pronase when broken membranes are digested: a new product which has lost a 10 000 dalton fragment is formed at very low pronase concentrations (as identified by its broad staining profile, Fig. 9), but this disappears at higher concentrations or during more extended treatment. It is unlikely that this new product arises from contaminating chromogranin A, rather than from dopamine β-hydroxylase itself: the major proteolytic degradation product of chromogranin A (which, although not shown in Fig. 9, can be seen in Fig. 8) in fact runs at a slightly lower molecular weight than this new band.

The glycoproteins of granules and membranes subjected to pronase digestion

were examined by staining gradient gels by the periodic acid-Schiff method. One of the stained regions (labelled B, Fig. 2) was susceptible to pronase when intact granules were digested: four additional bands were lost when broken membranes were treated (Table II).

Galactose oxidase and borohydride labelling

Intact granules and broken membranes were subjected to galactose oxidase treatment, followed by reduction with KB^3H_4. Following staining and scanning, gels of membrane proteins were sliced and their radioactivity assessed (Fig. 10). Several broad radioactive bands are seen when broken membranes are used, but intact granules do not appear to be susceptible. This result is in agreement with that of Huber et al. [10]. The positions of these bands were compared with the positions of glycoproteins as revealed by staining and by fluorescent lectin binding: the results are given in Table II. Some unusual bands are prominent in the Coomassie blue scan of Fig. 10. They appear to arise as a result of proteolytic activity associated with the galactose oxidase preparation.

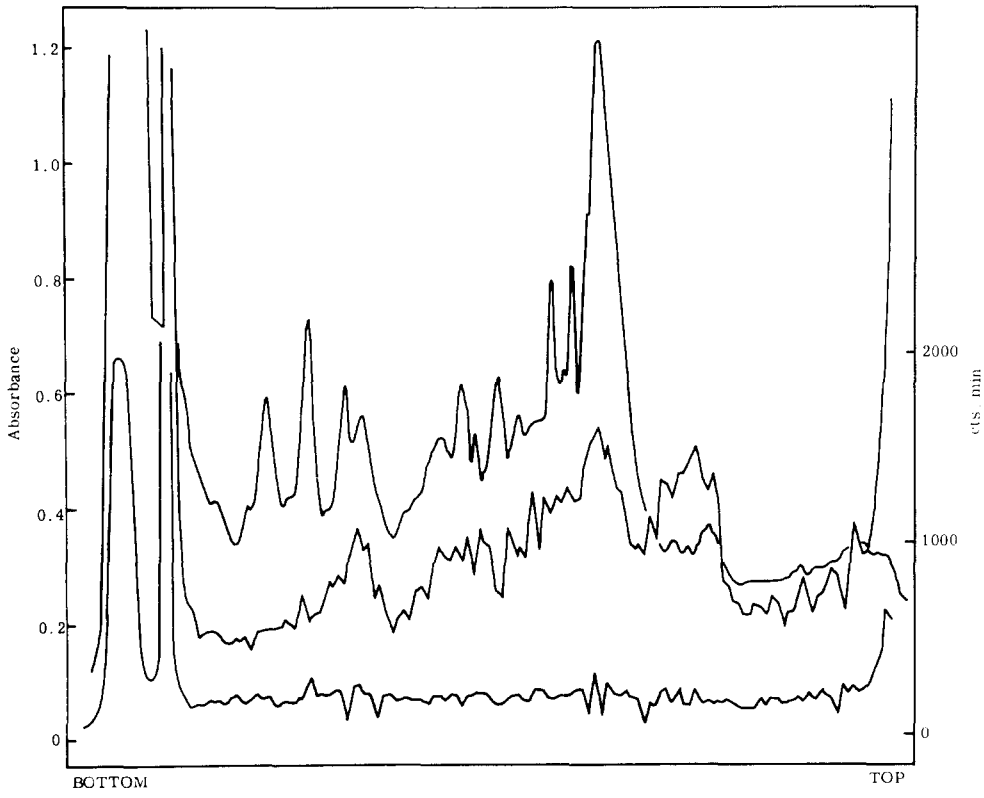

Fig. 10. Galactose oxidase treatment of granules and membranes. Intact chromaffin granules or broken membranes were treated with galactose oxidase followed by KB^3H_4 reduction. Purified washed membranes were then subjected to electrophoresis (8% gel). Following staining with Coomassie brilliant blue (upper trace) the gel was cut into 1 mm slices and radioactivity determined. Middle trace: radioactivity from treated broken membranes. Lower trace: radioactivity from treated intact granules.

Discussion

Polypeptides differ in their Coomassie blue-staining properties, in how sharply they focus and in how well they are resolved from each other, so that the 60 bands we observe on gels must of necessity be an underestimate. Presumably more would be resolved by two-dimensional techniques [39]. The particular difficulty that arises in our experiments is the probability that single bands on gels contain in reality more than one polypeptide, since this makes assignments of accessibility to reagents very hard to interpret. It has also made it difficult to unambiguously identify the same bands appearing on gels run under a variety of conditions, as, for example, in the comparison made between 8% gels and gradient gels in Fig. 1. We have examined gels run by a number of other workers: while major features of the band pattern are always recognisable, the fine detail is always different. In Fig. 1 we have therefore labelled many of the major and reproducible bands; in Table I we give apparent molecular weights, derived by reference to the positions of standards as shown in the figures, in order to enable others to correlate their results with ours. We also indicate those bands that are most prominent in the pattern. It can be seen (Table I) that molecular weights found on the two gel systems are in reasonable agreement: bands were lined up on the two gel systems by reference to their staining intensity as well as by their positions relative to standard markers.

Because some of the minor bands were not very reproducible in our hands, and may well arise from contamination of our membranes, we are mainly concerned with the major bands (about 40 in total) in the discussion that follows.

Comparison of labelling experiments

The crucial problem with experiments involving membrane labelling is the maintenance of the intactness of the organelle during the experiment. Because of the susceptibility of chromaffin granules to lysis, we purified them after labelling, having previously inactivated the reagent. We then monitored the internal proteins of the granules in order to check on the permeability of the membrane to the reagent. (These proteins are released by lysis during preparation of the granule membranes, and were studied by scanning gels on which they had been separated.) In no case was there evidence to suggest that the reagents used had penetrated the membrane under our reaction conditions.

We found that careful inspection of many gel patterns was needed in order to identify labelled or digested bands with the stained bands of control membranes. Gel slicing leads to artefactual background variation (see, for example, Figs. 3, 4 and 10) and analysis of several different membrane preparations was used in order to overcome this. In interpreting the pronase digestion experiments (Figs. 8 and 9), it is hard to distinguish between pronase-resistant polypeptides, and the appearance of bands due to the formation of new products. We varied both time of digestion and pronase concentration in order to resolve such difficulties as far as possible. We found with the pronase experiments that some bands were lost from the gels after a few minutes' digestion, while others were much less susceptible and remained, albeit at reduced intensity, after as long as 24 h. We have classified such bands as 'partially' sensitive (Table I),

TABLE I

LABELLING PROPERTIES OF BANDS

Coomassie brilliant blue (CBB)-staining intensity is indicated only if a band is a weakly staining (W) or a strongly staining major component (S) of the gel pattern; a blank indicates an intermediate intensity. Iodide/lactoperoxidase labelling and AEDANS labelling are indicated as follows: ++, major band; +, band clearly labelled in all experiments; (+), band usually labelled; —, band not labelled. Brackets link unresolved bands, and blanks indicate bands that could not be classified. Pronase sensitivity: +, pronase sensitive; —, pronase resistant; P, partial pronase sensitivity; blank, uncertain classification (sometimes due to the presence of new bands resulting from digestion).

Band number	Molecular weight ($\times 10^{-3}$)		CBB-staining intensity	Lactoperoxidase		Pronase		AEDANS	
	8% gel	8—20% gel		Granules	Membranes	Granules	Membranes	Granules	Membranes
1	134	135		—		+	+	++	++
2	132			+	+	+	+		
3a	127		W	+	+	—	+	+	+
3b	121	126		+	+	+	+	+	+
3c	113		W	+	+			+	++
4	107	116		+	+	+	+	—	—
5	103	109		+		+	+	+	+
6	100			—	+	—	+	+	+
7	98	102	W		—		+	(+)	(+)
8	96	100	W		+				
9	93	96	W	—	—	+	+	(+)	(+)
10	91	92		+		—	+		
11	88	89	W	—	(+)	—	+	+	+
12	86	87		+	+	+	+	+	+
13	79	81	S	+	++	—	+	+	+
14	77	78	S	+	+	—	+	+	+
15	74	74	S	+	+	—	+	+	++
16	71	69	S	+	++	—	+	++	++
17	70		S		(+)	—	+		
18	69	68		—	+	+	+	+	+
19	68	66		+	+	+	+		
20	66	65	W	—	—	+		+	+
21	65	63	W		+	—			
22	63	61		—	++	P	+	—	+
23	61		W			+			
24	60	58	S	+	++	P	+	++	++
25	59	56	W	+	+	P	+		
26	58	55	S	++	++	+	+	++	++
27	56		W	(+)	(+)		+	+	+
28	54	53		+	+	P	+	+	+
29	53	52				P	+	+	+
30	51	49		+	+	P	+	++	++
31	50			+				+	+
32	48	47		—	+	—	+	(+)	+
33	47	46	W	(+)	—			+	+
34	46	44		—	(+)	P	P	+	+
35	44	42	W	(+)	—	+	+	+	+
36	42	41	W	+	+	+		(+)	(+)
37	40		W	+	+				
38	39	38	S	—	++			++	++
39	38	36	S	+	++	+	+	++	++
40	37	35	W	+	+			+	+
41	34	34			(+)	+	+	++	++
42	34	33	S			+	+		
43	33	33	W	+	+			(+)	(+)
44	31	30		+		+		(+)	(+)
45	30	29		(+)		+			

TABLE I (continued)

Band number	Molecular weight ($\times 10^{-3}$)		CBB-staining intensity	Lactoperoxidase		Pronase		AEDANS	
	8% gel	8–20% gel		Granules	Membranes	Granules	Membranes	Granules	Membranes
46	29	28	W	++	++	—	—	++	++
47	27	27	W			+			
48	26	25	W	+	+	P	+	(+)	(+)
49	25	24		++	+	P	+	++	++
50	23	23		—	+	P	+	—	+
51	22	22	S	+		+	+	+	+
52		22	W			—	+		
53		21				+	+		
54		20				+	+		
55		19.5	W						
56		18.5				+	+		
57		18	W			+	+		
58		17				+			
59		16.5	W			+			
60		14				+	+		
61		13.3	S			—	—		
62		12.8	W			+	+		
63		12.0	W			+	+		
64		11.4				—	—		
65		10.7	W			+	+		
66		10.0	W						

implying that they may represent two or more polypeptides (one sensitive and one resistant to pronase), as well as single polypeptides which are hydrolysed very slowly.

Not all bands are susceptible to labelling with lactoperoxidase or 1,5-AEDANS, or to digestion with pronase, when broken membranes are used. Allowing for this, the most striking result of our experiments is the high proportion of bands that are susceptible when intact granules are treated. The patterns from granules and from broken membranes were not reliably distinguishable in the case of 1,5-AEDANS labelling; only five minor bands consistently showed more fluorescence when broken membranes were used. With iodide and lactoperoxidase, 33 out of about 40 susceptible bands are labelled in intact granules, and with pronase 28 out of 46 susceptible bands are digested. We conclude that a high proportion of chromaffin granule membrane polypeptides are exposed on the outer (cytoplasmic) surface of the granule.

By contrast, rather few are exposed solely at the inner surface (as identified by the criterion that they are susceptible in broken membranes but not in granules). Approximately five such components can be identified from the 1,5-AEDANS experiments, nine from iodide/lactoperoxidase experiments and 18 from the pronase experiments. The fact that the three techniques do not identify identical sets of bands may be due to the different properties of the probes: the first two clearly require accessible cysteine and tyrosine residues for effective labelling, while the third may be highly dependent on protein conformation. Major features identified by these methods are discussed below.

Membrane glycoproteins

We list in Table II groups of Coomassie blue-stained bands which appear to comigrate with glycoprotein bands, without implying (except as discussed below) that any of these are themselves glycoproteins. Most of the glycoproteins are labelled by galactose oxidase and KB^3H_4, but only when broken membranes are utilised. Intact granules are not labelled. This confirms the conclusion of Huber et al. [10] that the carbohydrate residues of most of the glycoproteins are located on the matrix side of the membrane and do not face the cytoplasm. It also fits with the electron microscopic observations of concanavalin A binding to the inner surface of chromaffin granule membranes [9, 10]. Meyer and Burger [40] have reported the existence of protein-bound wheat germ agglutinin receptors (presumably *N*-acetylglucosamine) on the cytoplasmic surface of the granule and, in addition, sialic acid (possibly as a component of glycolipid) has been reported on the outer surface [30,41]. These residues have not been revealed by our experiments.

One glycoprotein band revealed by the periodic acid-Schiff method (B of Table II, about 95 000 daltons) is susceptible to pronase when intact granules are digested. Since this band is labelled by the galactose oxidase procedure only when broken membranes are treated, we can tentatively conclude that its polypeptide portion spans the membrane and is exposed on the cytoplasmic surface.

Several of the glycoproteins (bands A, E, F, G and H) are not susceptible to pronase in the broken membranes. This enables us to conclude that they are

TABLE II

GLYCOPROTEINS OF CHROMAFFIN GRANULE MEMBRANES

Glycoprotein bands are identified by staining with dansyl hydrazine and with fluorescent lectins as well as periodic acid-Schiff (PAS) staining (data not presented; see Cahill and Morris [11]). Approximate molecular weights of corresponding Coomassie brilliant blue (CBB)-stained bands are given. Intensities of PAS staining and galactose oxidase/KB^3H_4 labelling are shown as + or ++ (S, a shoulder on another peak; —, lack of labelling). Our data for fluorescein-concanavalin A (con A) labelling of bands in gels (Con A column) agree closely with those of Cahill and Morris [11], where data for other lectins are given. Pronase sensitivity (—, pronase resistance) was assessed on PAS-stained gels only.

—	CBB band numbers	Molecular weight ($\times 10^{-3}$)	PAS staining	Pronase sensitivity		Galactose oxidase labelling (membranes)	Con A
				Granules	Membranes		
A	5,6	103	+S	—	—	++	++
B	7—10	95	++	+	+	++	
C	13—15	77	+S	—	+	+S	++
D	16—18	69	++	—	+	++	
E	22	63	+	—	—	++	
F	24—26	59	+	—	—	+S	++
G	26—30	56	+S	—	—	+	++
H	30—34	49	++			+	++
I	38	39	++	—	+	++	+
J	39	37				+	
K	41,42	34	—			—	+
L	44,45	30	—			—	+
M	47,48	26	—			—	+

therefore not generally identical with corresponding Coomassie blue-stained bands, which are invariably digested by pronase under these conditions.

Major features of chromaffin granule membrane polypeptides

The results of all the labelling experiments are summarised in Table I. They suggest that some polypeptides are primarily exposed only at the matrix surface of the membrane. Bands 13—15, for example, are all exposed in this way. They are broad bands (containing the glycoprotein dopamine β-hydroxylase) superimposed on other sharp bands that are revealed in gels of non-reduced material that are run in parallel. Although they are not very susceptible to labelling with 1,5-AEDANS, the lactoperoxidase and pronase results are unequivocal, with greatly increased labelling and digestion occurring on granule lysis.

Other bands which appear to be exposed only on the inner (matrix) surface are bands 6, 16, 17, 22, 24 (which probably contains two polypeptides, one of which is exposed on the outer surface), 32, 38 and 50; about thirty bands are definitely exposed on the cytoplasmic surface. The evidence is equivocal for other bands.

Bands 28 and 29, a close doublet of just over 50 000 daltons, sometimes comigrate on 8% gels, although these bands are better separated on gradient gels. They correspond to the α and β subunits of the ATPase, as we have shown by partially purifying this enzyme according to the procedure of Apps and Glover [25]. Surprisingly, although enzymic activity is lost rapidly when intact granules are digested with pronase (Fig. 7: 90% of activity is lost within 1 h), these bands are not lost from the gel so rapidly (although they are degraded by pronase within 10 min when broken membranes are treated). After extensive digestion of intact granules, bands 28 and 29 still appear as a characteristic doublet, only slightly reduced in intensity as compared with the control. Possibly other components comigrate here, or only a small fragment is removed by pronase (although membrane disruption leads to complete sensitivity of the bands). Band 30 (about 48 000 daltons) has similar properties with respect to pronase, although the other reagents clearly label it well in intact granules.

A characteristic doublet appears at about 35—38 000 daltons (bands 38 and 39). These bands are very strongly labelled by lactoperoxidase treatment of broken membranes, while band 38 is protected in intact granules. Unfortunately its pronase sensitivity is hard to assess as new products are prominent in this region of the gel. It is almost certainly a glycoprotein; it comigrates with glycoprotein I (Fig. 2), and often appears as a rather broad band when stained with Coomassie blue. It is susceptible to galactose oxidase labelling when the membranes are broken. The only uncertainty with respect to band 38 is the fact that it is a major site of 1,5-AEDANS labelling in intact granules as well as in broken membranes.

Bands 49—51 tend to comigrate on high percentage gels to give a very intense band which has been named 'chromomembrin B' by Hörtnagl et al. [35]. These bands are clearly separated on 8% gels (Fig. 1). Band 49 has the unusual property of being much more heavily labelled by iodide/lactoperoxidase in granules than in broken membranes (Fig. 3): we have no clear explana-

tion for this phenomenon. The major band of this area is band 51 (21 000 daltons); this is of interest since pronase digestion of intact membranes gives rise to a product of 18 000 daltons which is not digested further, whereas digestion of broken membranes completely eliminates this product. It seems likely, therefore, that this component is not only exposed on the outer surface, as found by König et al. [7], but also spans the membrane, like glycoprotein B, discussed above. In general, however, these experiments do not give information about such proteins, since they will be classified as appearing on the cytoplasmic surface.

Some polypeptides are resistant to the reagents we have used, since we have concentrated on reagents acting at the exposed surfaces of the membrane. Preliminary experiments with hydrophobic reagents (Apps, D.K. and Geisow, M., personal communications) reveal that a different set of polypeptides can then become labelled.

This study of an intracellular membrane is consistent with other studies on plasma membranes, showing that most proteins are exposed on the cytoplasmic face, but that most carbohydrate residues are on the opposite surface of the membrane.

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