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TWO-DIMENSIONAL GEL ELECTROPHORESIS OF RAT LIVER MICROSOMAL MEMBRANE PROTEINS

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Summary

Total rat liver microsomal proteins are not suitable for isoelectric focusing in polyacrylamide gels, even in the presence of sodium dodecyl sulphate and excess non-ionic detergent; considerable quantities of protein form an aggregate in the isoelectric focusing gel. This prevents resolution of microsomal proteins by the increasingly popular two-dimensional electrophoresis technique employing isoelectric focusing followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The problem is caused by the extreme insolubility of some microsomal proteins, especially cytochrome *P*-450 species, which precipitate during isoelectric focusing. A selective extraction of microsomes with sodium deoxycholate excludes these poorly soluble proteins. The extracted proteins can then be resolved without difficulty by isoelectric focusing, and give excellent two-dimensional gel patterns showing more than 100 proteins, mainly in the p*I* range 5—7. The technique should be useful in studies on microsome protein topology and on changes in microsome composition.

Introduction

Despite intensive study, there are still considerable gaps in our knowledge of the protein composition of microsomal membranes and the organisation of these components in the endoplasmic reticulum membrane [1,2]. As a result, we know little of the molecular basis of the numerous functions of this significant organelle, including lipid metabolism, activation and modification of xenobiotic compounds, ribosome binding and the post-translational modification of secretory proteins. In other systems, considerable advances have followed from the introduction of reproducible techniques for resolving,

enumerating and distinguishing the protein components of an organelle. Thus, recent advances in our understanding of the organization and function of the erythrocyte membrane followed the introduction of sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis for resolution of the membrane proteins [3,4]. In a more complex system, the bacterial ribosome, the use of a two-dimensional gel electrophoresis technique [5] and the adoption of a standard nomenclature based on this technique by many workers in the field [6] greatly accelerated progress.

The most powerful widely-applicable technique for resolution of a large number of proteins is the two-dimensional technique of O'Farrell [7] which employs isoelectric focusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension. This has subsequently been modified for use with membrane proteins [8] and is proving to be of wide applicability despite problems associated with the poor solubility of some membrane proteins in the non-ionic detergents used in the isoelectric focusing gel. This technique is particularly attractive for resolution of microsomal proteins because of the large number of major protein components in the molecular weight range 40 000—60 000 and the consequent poor resolution of these proteins in standard one-dimensional SDS-polyacrylamide gel electrophoresis [9—11].

We describe here the resolution of over 100 rat liver microsomal membrane proteins by a modification of the technique of Ames and Nikaido [8]. Modification was necessary because of problems caused by the extreme insolubility of cytochrome *P*-450 species under the conditions of isoelectric focusing. It has recently been noted that these highly hydrophobic proteins are liable to aggregation during isoelectric focusing in polyacrylamide and granulated gels [12]. We describe a detergent (sodium deoxycholate) extraction technique which selectively excludes these poorly soluble proteins, and allows complete resolution of the extracted microsomal membrane proteins. Some preliminary results have already been presented [13].

Methods

Materials

All chemicals employed were of analytical grade quality. Acrylamide and *N*,*N*-methylene bisacrylamide (specially purified for electrophoresis), urea (Aristar grade), sodium dodecyl sulphate (specially purified for biochemical work) and NP-40 were purchased from BDH Chemicals Ltd. pH gradients in the first dimension were produced by Ampholines, range 3.5—10, 4—6, 6—8, 40% (w/v), obtained from LKB. The following proteins were used to calibrate the second-dimension gel (molecular weight in parentheses): bovine serum albumin (68 000) from BDH chemicals, glutamate dehydrogenase (53 000) and aldolase (40 000) from Boehringer Corpn. (London) Ltd, and β-galactosidase (130 000), phosphorylase *a* (94 000), ovalbumin (45 000), haemoglobin (17 000) and cytochrome *c* (13 000) from Sigma Chemical Co. Other chemicals were also obtained from Sigma Chemical Co.

Microsome preparation

Microsomes were prepared from livers of male, Sprague-Dawley rats (150—

200 g), which were starved for at least 8 h to deplete glycogen in the liver. The rats were killed by cervical fracture, the livers removed immediately, weighed and washed repeatedly in cold buffer 1 (0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM KCl) to remove blood and other debris. All subsequent procedures were carried out at 4°C. After dissecting off the connective tissue, the organ was chilled and homogenised in a Teflon-glass Potter motor-driven homogeniser. 1 g of liver was homogenised in 1.5 ml of buffer 1. The homogenate was centrifuged for 10 min at 600 × g_{av} to remove nuclei and unbroken cells. A post-mitochondrial supernatant was obtained from the supernatant of the first spin by centrifugation for 10 min at 13 000 × g_{av}. The application of the two-stage centrifugation procedure is important for the removal of the 'white fluffy' layer which floats at the supernatant surface. The 'white fluffy' layer has a strong attraction for microsomal membranes and proves to be a contaminant in further stages of purification of microsomes. The microsomal fraction was prepared from this post-mitochondrial supernatant by gel filtration on Sepharose 2B [14,15]. 50 ml of post-mitochondrial supernatant were applied over a 300 ml bed of the gel (dimension 4.5 cm internal diameter and 18.5 cm height), pre-equilibrated in buffer 1. The gel-filtered microsomal fraction from the void volume was then harvested by centrifugation for 1.5 h at 105 000 × g_{av}. The retarded protein peak representing the soluble cytosolic proteins was also retained.

Microsomes from phenobarbital and 3-methylcholanthrene-treated rats were kindly provided by Dr. P.R. McIntosh.

Degranulation was carried out by suspending the gel-filtered microsomal fraction in the presence of puromycin and 0.5 M KCl [16], incubating for 1.5 h and centrifuging on a discontinuous sucrose gradient for 3 h at 105 000 × g_{av}. The degranulated microsomal fraction was isolated from the 0.25—1.35 M sucrose interface. Delipidation was carried out using the acetone extraction as described in Ref. 17.

Protein concentrations were estimated by using the method of Lowry et al. [18], using bovine serum albumin as standard.

Solubilisation of microsomal membrane proteins by deoxycholate

Freshly-prepared microsomes, resuspended in buffer 1, at approx. 15 mg/ml protein concentrations were subjected to deoxycholate treatment in the range between 0 and 0.3% (w/v) as the final concentration of the detergent in the suspension. The sequence of additions was water to microsomes followed by the detergent. During the addition of detergent the suspensions were mixed in a vortex mixer and afterwards allowed to incubate on ice for 30 min. The mixture was centrifuged for 1.5 h at 105 000 × g_{av} yielding a pellet and pale-yellow supernatant. The supernatant was collected and the pellet was resuspended in buffer 1. Both the fractions from each point in the titration were retained for examination in sodium dodecyl sulphate-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis.

Sample preparation for isoelectric focusing

Except where stated otherwise, the following protocol was adopted for preparation of sample for application in isoelectric focusing. A 2 ml aliquot of

suspension at approx. 4—6 mg/ml protein concentration was treated with 50 μl of RNAase A and 50 μl of DNAase I, each at 4 mg/ml protein concentration, and allowed to incubate at room temperature for 30 min. Digestion of nucleic acids in the mixture was ascertained by monitoring an increase in absorbance at 260 nm, after addition of the nucleases. For solubilisation, a 0.4 ml aliquot of this mixture was added to 50 μl sodium dodecyl sulphate (10%, w/v). Additional solubilisation was achieved by heating the sample at 70°C for 30 min. The sample was brought to 9 M urea and 1% SDS by dialysing against 50 ml of 9 M urea and 1% SDS. The sample was used immediately as storage of the sample for long periods leads to artefactual heterogeneity in isoelectric focusing. Immediately before electrofocusing, the sample was diluted with an equal volume of isoelectric focusing sample dilution buffer containing 9 M urea, 5% (v/v) β-mercaptoethanol, 8% (w/v) NP-40, 0.5% (w/v) Ampholines (pH range 3.5—10). 50—100 μl of sample containing 100—300 μg of protein were loaded at the basic end by displacing sample overlay (150 μl isoelectric focusing sample dilution buffer plus 50 μl water) previously placed over the gel. Whenever the sample was loaded at the acidic end of the gel, both the electrode polarities and the reservoir electrolytes were reversed.

Isoelectric focusing was performed in cylindrical gels (2.7 mm internal diameter and 10—11 cm long) essentially as described in Ref. 8. Unless stated otherwise, the composition of the isoelectric focusing gels was 5% (w/v) acrylamide, 9 M urea, 2% (w/v) NP-40, 2% (w/v) Ampholines. Polymerisation of the gels was initiated by adding 40 μl of 10% (w/v) ammonium persulphate to 10 ml of mixture. The total isoelectric focusing period was restricted to 5000 V · h. The cylindrical gels were then subjected to SDS-polyacrylamide gel electrophoresis at right angles by being incorporated into an agarose layer composed of 1% (w/v) Agarose, 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) sodium dodecyl sulphate, 0.0625 M Tris-HCl (pH 6.8) over a 1 cm stacking gel which in turn was layered over a sodium dodecyl sulphate-polyacrylamide separation gel. Electrophoresis was operated at a fixed current of 30 mA and variable voltage (maximum 250 V) for 5—6 h. The method used was that of O'Farrell [7] except for the use of homogeneous separating gels in some cases. Gels were stained with 0.25% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid, and then destained in several changes of destaining solution (30% (v/v) ethanol, 10% (w/v) acetic acid). The gels were photographed through an ultraviolet filter over Ilford Pan F film.

Sample preparation for SDS-polyacrylamide gel electrophoresis

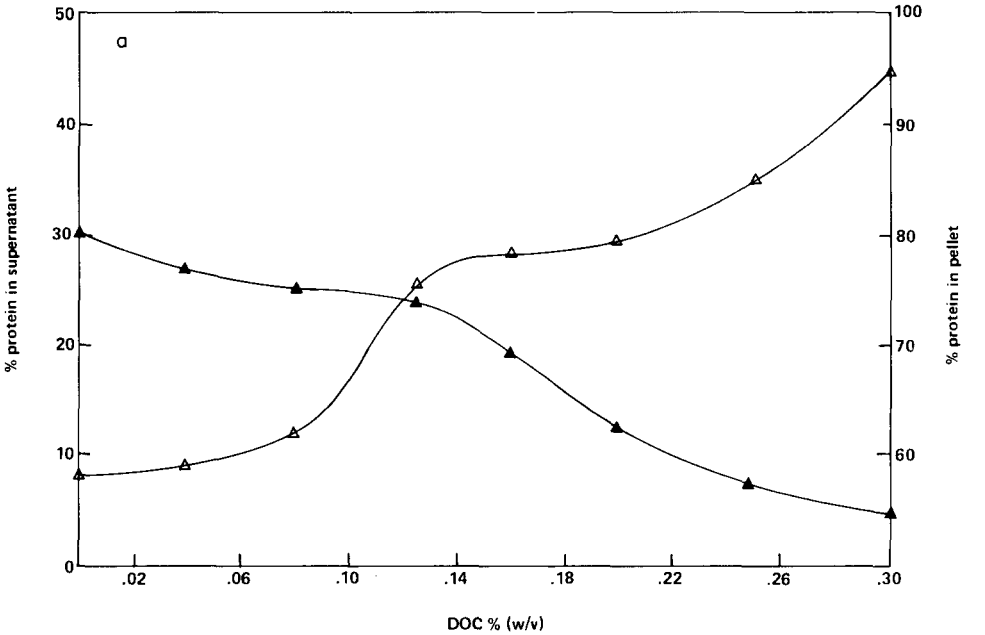
For samples to be run in one-dimensional SDS-polyacrylamide gel electrophoresis, 200 μl of protein suspension were mixed with 100 μl of SDS sample dilution buffer composed of 5% (w/v) SDS, 12.5% (v/v) β-mercaptoethanol, 33% (v/v) glycerol. The mixture was heated at 70°C for 30 min. A required volume containing approx. 100 μg of proteins was loaded into the well of the slab gel.

Results

The isoelectric focusing method developed for membrane proteins by Ames and Nikaido [8], which employs solubilization in SDS followed by addition of excess non-ionic detergent, was generally unsuccessful in resolving rat liver microsomal membrane proteins. Some proteins entered the gel and gave well-resolved bands, but the bulk of material applied formed an unresolved region near the point of application (see, e.g., Fig. 2c). In favourable cases, sufficient protein entered the isoelectric focusing gel for subsequent resolution by SDS-polyacrylamide gel electrophoresis to be practicable. The small number of proteins clearly resolved gave a characteristic pattern of spots in two dimensions, but a large proportion of the protein either remained at the origin, or was only resolved in the second dimension, so forming a strong band down one side of the slab gel, with streaks running into the gel. Comparable problems were not encountered in the two-dimensional resolution of cytosol proteins.

A slight improvement was effected by using the dialysis technique of Peterson and McConkey [19] for addition of urea during sample preparation, possibly because of the removal of buffer ions [20]. No improvement was produced by any of the following treatments of the sample: (i) degranulation with KCl-puromycin; (ii) extensive digestion with nucleases; (iii) acetone extraction of lipids; (iv) centrifugation of the final sample for 3 h at 250 000 $\times$ g_{av}. Experiments with large pore gels (4% acrylamide), with loading at the acidic electrode and with incorporation of the sample into the gel mix before polymerization, were all unsuccessful. Other polyoxyethyleneglycol detergents such as Triton X-100 and Triton N-101 could be used interchangeably with NP-40 but gave no improvement in resolution.

These findings indicate that some protein component(s) of microsomal membranes, though soluble and not sedimentable in the loaded sample, come out of solution during the initial period of isoelectric focusing, which removes SDS from the basic end of the gel [21]. These components are presumably not soluble in non-ionic detergents alone, which suggested that they could be excluded by a selective detergent-extraction procedure. A detailed study of the solubilization of microsomal proteins was carried out by Kreibich et al. [10,22], who showed that both the detergent concentration and the detergent : protein ratio were critical factors. Their most widely exploited result was the demonstration that under defined conditions (0.05% (w/v) deoxycholate and at about 5 mg/ml protein), luminal content proteins are released without solubilization of the membrane. Nevertheless, their results also show that at higher detergent concentrations, most membrane components are solubilized, but that ribosomal proteins and major proteins of molecular weight approx. 45 000 remain sedimentable (see, e.g., Ref 10, Fig. 1b). Likewise, we have found that microsomal membrane proteins may be selectively solubilized with sodium deoxycholate and Fig. 1 shows the extent of solubilization as a function of detergent concentration, and the SDS-polyacrylamide gel electrophoresis patterns of the solubilized proteins. It is clear that the heavy bands (dots) at the lower molecular weight end of the central heavily staining region only appear in samples solubilized with very high detergent concentrations.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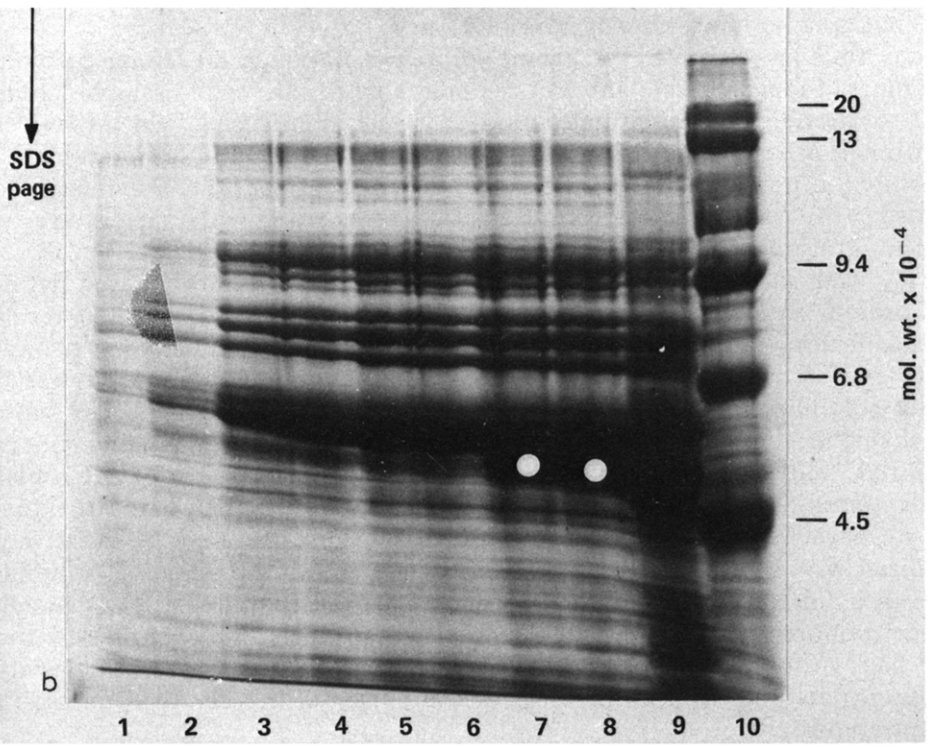
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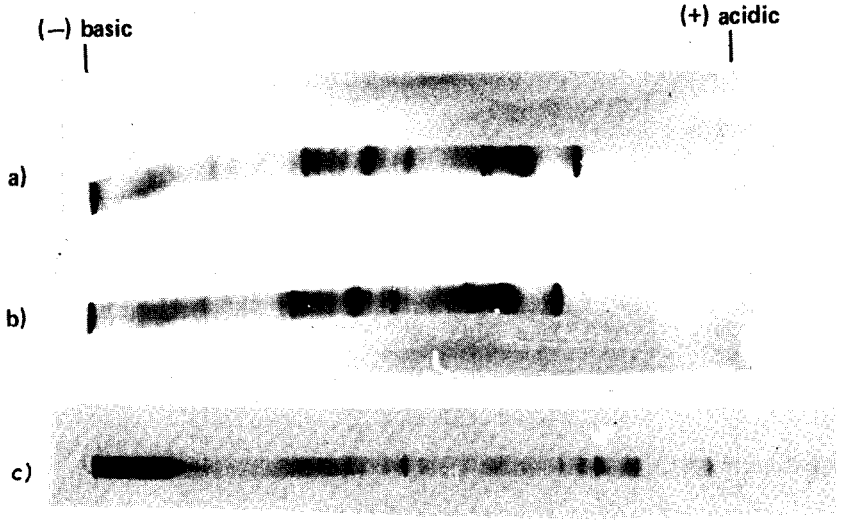
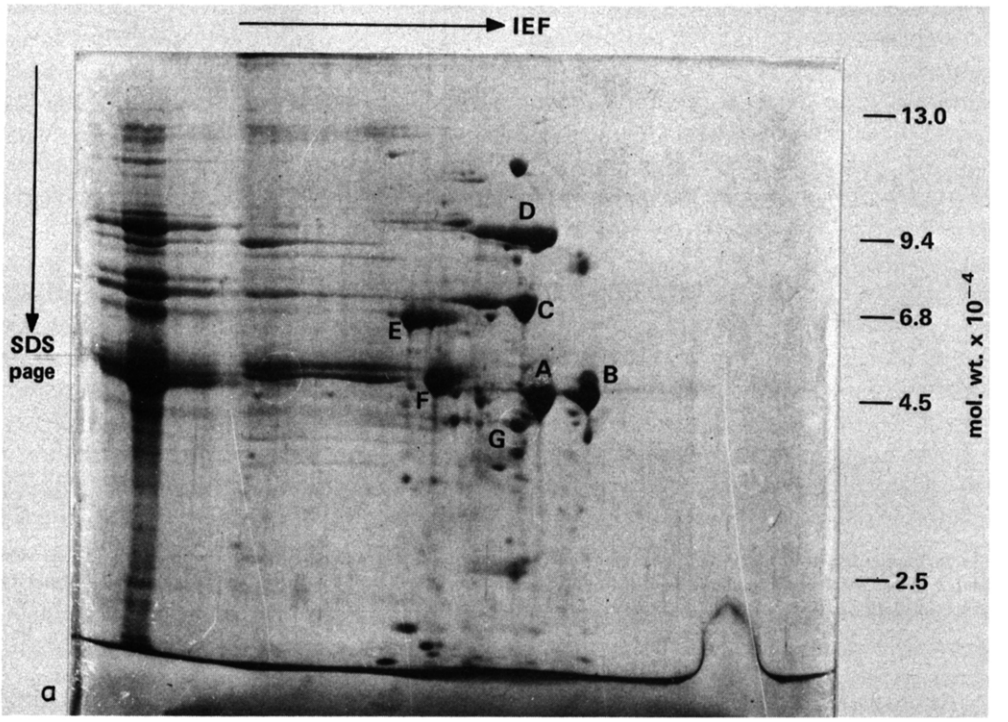


Fig. 2. Isoelectric focusing of microsomal membrane proteins extracted with deoxycholate. Samples were prepared for isoelectric focusing as described in Methods. 100—150 μg of proteins were applied. (a) 0.15% deoxycholate extract; (b) 0.2% deoxycholate extract; (c) total microsomal proteins.

At lower detergent levels (approx. 0.2%, w/v), the proteins responsible for these bands are not solubilized, and the remaining proteins extracted at this detergent concentration can subsequently be subjected to isoelectric focusing with very little aggregation (Fig. 2). Such deoxycholate extracts therefore give rise to excellent two-dimensional patterns in which very little protein has failed to enter the gel and be resolved (Fig. 3). In contrast, the deoxycholate-insoluble pellet fractions failed to be resolved in isoelectric focusing (data not shown). With the deoxycholate-extraction technique, the elimination of aggregation and the decrease in streaking allow more protein to be loaded, so that many more protein spots can be clearly distinguished. The use of gradients of polyacrylamide concentration in the second dimension slab gels improves the resolution of low molecular weight proteins (Fig. 3b). The pattern of spots is highly reproducible provided that samples are not stored in urea for long periods before running; long periods of exposure to

Fig. 1. (a) Protein content in supernatant and pellet fractions from deoxycholate-treated microsomes. Microsomal membranes (15 mg/ml) were subjected to a range of detergent treatments (0—0.3%, w/v) by addition of 2% (w/v) sodium deoxycholate (DOC); final volume 2 ml. After centrifugation, protein was determined in both pellet (▲———▲) and supernatant (△———△) fractions. (b) Supernatants from deoxycholate-treated microsomes, resolved in 10% SDS-polyacrylamide gel electrophoresis (SDS page). 1, 0% deoxycholate (control); 2, 0.04%; 3, 0.08%; 4, 0.12%; 5, 0.16%; 6, 0.20%; 7, 0.25%; 8, 0.3% deoxycholate; 9, total microsomal proteins; 10, marker proteins (molecular weight in parentheses) myosin (200 000), β-galactosidase (130 000), phosphorylase (94 000), bovine serum albumin (68 000), ovalbumin (45 000). 200 μl of each of the deoxycholate extracts were mixed with 100 μl of SDS sample dilution buffer and the mixture heated at 70° C for 30 min. A required volume containing approx. 100 μg was applied for electrophoresis, except in 1 and 2 where the maximum possible volume (200 μl) was applied. The white spot marks the protein component(s) in the region of cytochrome *P*-450, molecular weight 48 000—52 000 daltons, released from the membrane only at deoxycholate concentration greater than 0.25% (w/v).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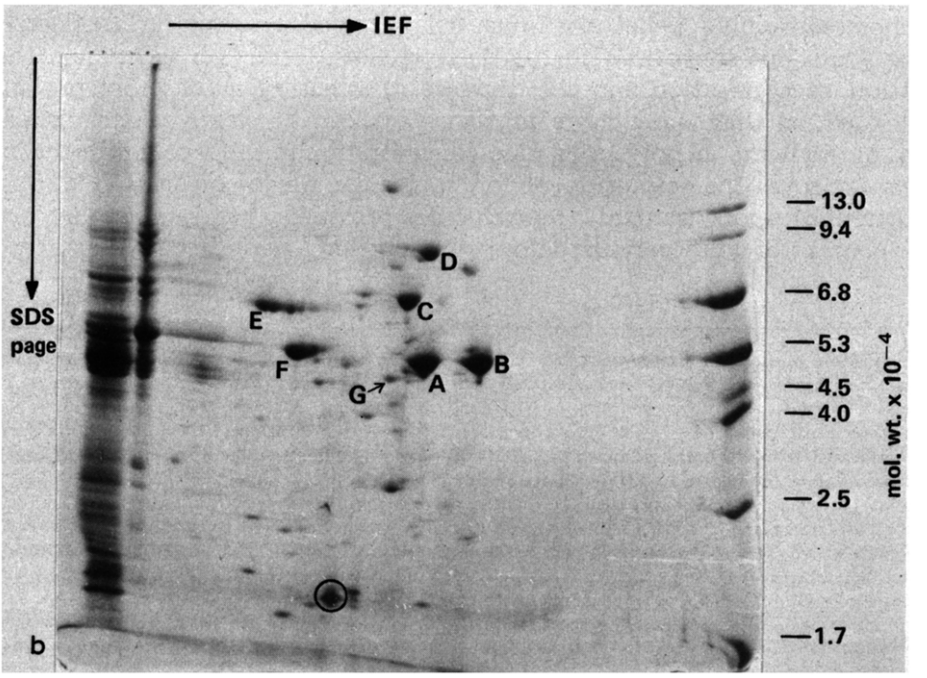
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urea led to the appearance of characteristic families of spots, presumably as a result of carbamylation [7,23].

Discussion

Difficulties caused by the poor solubility of membrane proteins under conditions suitable for isoelectric focusing and two-dimensional gel electrophoresis have been discussed previously [8,21]. Our data show that these problems are particularly acute with rat liver microsomal membranes. Several lines of evidence suggest that cytochrome *P*-450 species are mainly responsible. (1) In two-dimensional gels of total microsomal proteins, aggregation and streaking were most marked in the region of the gel around 48—52 kilodaltons, corresponding to the molecular weights of the known rat liver cytochrome *P*-450 species [24—26]. (2) Microsomes from rats in which high levels of cytochrome *P*-450 had been induced by pretreatment with phenobarbital showed very severe aggregation and no resolution of any proteins on isoelectric focusing of whole microsomes. (3) Total microsomes from rats pretreated with 3-methylcholanthrene, and known to contain specific new cytochrome *P*-450 species (on the basis of standard SDS-polyacrylamide gel electrophoresis) showed a similar pattern to total control microsomes on two-dimensional electrophoresis, implying that the induced species were not appearing on the two-dimensional gel. (4) Cytochrome *P*-450 species are known to be highly hydrophobic proteins, and a recent paper has shown that they are very poorly resolved on isoelectric focusing in polyacrylamide and agarose gels [12].

The selective extraction procedure described here leaves in the residual pellet specific proteins of molecular weight 48—52 kilodaltons; on the grounds of their insolubility, their molecular weights, and the large proportion which they form of the total microsomal protein, we conclude that these proteins are cytochrome *P*-450 species. In their absence, the extracted proteins are well resolved by isoelectric focusing and two-dimensional electrophoresis. The primary drawback of this approach is that the final two-dimensional gel does not represent all the protein components of microsomal membranes, since some are deliberately excluded by their poor solubility. The major proteins absent are almost certainly cytochrome *P*-450 species and ribosomal proteins (both because of their low solubility in deoxycholate solutions [10] and because of their very basic isoelectric points). But other proteins may also be absent; we have preliminary evidence that epoxide hydratase is not extracted under these conditions.

Despite this disadvantage, the approach is useful and promises to extend considerably our knowledge of microsomal proteins. The resultant two-dimensional gels show that microsomal membranes contain more than 100 proteins. Previous work based on one-dimensional gel electrophoresis had led to esti-

Fig. 3. Two-dimensional resolution of microsomal proteins extracted with deoxycholate (0.2%). (a) Ampholine range in first dimension, pH 3.5—10; second dimension 10% (w/v) homogeneous polyacrylamide. IEF, isoelectric focusing. (b) Ampholine range in first dimension, pH 3.5—10 : 6—8 : 4—6 in the ratio 1 : 2 : 2. Second dimension 7—16% (w/v) linear gradient polyacrylamide gel. The major proteins marked A-G are definitely microsomal in origin and are not seen in comparable gels of cytosol fractions (not shown).

mates in which the number of proteins resolved had gradually increased from less than 20 [27] to more than 30 [9], to 51 [10] to 55—60 [11]. The proteins resolved by the two-dimensional procedure described here form a complex pattern dominated by a characteristic arrangement of seven major species. The patterns shows that the majority of microsomal proteins are mildly acidic with p*I* values in the range 5—7, consistent with the comparatively high level of aspartic and glutamic acids in microsomal membrane proteins [28]. For the following reasons, the estimate of 100 proteins is almost certainly a minimum figure. (1) The number of spots is limited by the sensitivity of Coomassie blue staining; radioactive labelling with high specific activity [^{35}S]methionine [29] might reveal further components. (2) The microsomes used here are prepared by gel-filtration and are not contaminated by cytoplasmic proteins; microsomes prepared by ultracentrifugation show a considerable number of additional faint spots [13]. (3) It is unlikely that any of the spots are artefacts of isoelectric focusing, since there are few of the characteristic horizontal families of spots produced by Ampholine binding or modification by urea [23]; some of the spots may represent variants of a single polypeptide differing in post-translational modifications (e.g., possibly species E) but we have no evidence on this point. (4) Finally, the high reproducibility of the pattern implies the absence of preparative and procedural artefacts.

Of course, 'microsomal membranes' do not represent a single organelle, and fragments of plasma membrane, Golgi membrane and nuclear membrane are probably present. Nevertheless, the material analysed here is what is generally referred to as the microsomal membrane fraction, and its major components are undoubtedly vesicles derived from the endoplasmic reticulum.

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