'map' that contained a large number (13) of these intensely staining spots against a low level of background staining. Tryptic-peptide 'maps' of spectrin (Marchesi *et al.* 1970), the major protein removed from the 'ghosts', showed a very large number of peptides that were poorly resolved from each other, and also contained the small number of the intense spots that were not present in 'maps' of salt-extracted 'ghosts'.

The salt-extracted 'ghosts' were chromatographed on Sephadex G-150 in the presence of 1% sodium dodecyl sulphate. The E_{280} pattern showed a broad spread of protein with a peak of material at 65 000–25 000 molecular weight, and a peak of material in the range 12 000–6 000 molecular weight. Amino acid analysis showed differences between the fractions. Carbohydrate analysis showed that the higher-molecular-weight peak was associated with high contents of galactose (260 nmol/mg), *N*-acetylglucosamine (220 nmol/mg) and sialic acid (300 nmol/mg), this material being nearly 30% carbohydrate by weight. The low-molecular-weight peak contained large amounts of *N*-acetylgalactosamine (230 nmol/mg), galactose (270 nmol/mg) and lipid.

The two protein peaks gave simple but different tryptic-peptide 'maps'. The positions of the peptides suggested that a major component of the higher-molecular-weight peak was not present in the low-molecular-weight peak, but did not exclude the possibility that the polypeptide present in the low-molecular-weight peak was also present in the high-molecular-weight peak. 'Fingerprints' of thermolysin digests of the material of the two peaks showed that a polypeptide component was indeed present in both peaks.

Digestion of the material of the low-molecular-weight peaks by carboxypeptidases A and B in the presence of sodium dodecyl sulphate released threonine and valine in approximately equimolar amounts (1 residue/200 residues of protein), with a slightly lower yield of alanine and leucine. Kinetic experiments suggested that the order of release was valine, threonine, alanine, leucine. It was not possible to distinguish between a single polypeptide with a *C*-terminal sequence of -Leu-Ala-Thr-Val and three or four chains with differing *C*-termini present in similar molar amounts. The higher-molecular-weight peak showed a similar pattern of release of the same amino acids in high yield (threonine, 1.3 residues/200 residues), serine being the only additional amino acid released (0.33 residue/200 residues). Each of the fractions obtained from the gel-filtration column showed the release of threonine, valine, alanine and leucine. The kinetics of the release were very similar for each fraction and the magnitude of the yields followed the order threonine > valine > alanine > leucine.

Salt-extracted 'ghosts' appear to contain two major groups of polypeptides. One of these, present in the higher-molecular-weight peak, is probably identical with the sialoglycoprotein isolated by several workers (Springer, Fletcher & Pavlovskis, 1967; Blumenfeld, Gallop, Howe & Lee, 1970). In addition, a distinct low-molecular-weight component aggregates throughout a wide molecular-weight range in 1% sodium dodecyl sulphate, and may be composed of a single polypeptide chain or a very small number of polypeptide chains that aggregate in such a way that the composition of the complex is the same irrespective of its molecular weight. This component is probably similar to the membrane miniprotein described by Laico *et al.* (1970).

We acknowledge the financial assistance of the Medical Research Council.

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Some Properties of Mitochondrial Membrane Proteins Dissolved in Chloroform-Methanol

By P. J. CURTIS. (*The Beatson Institute for Cancer Research, Glasgow C.3, U.K.*)

There is considerable experimental evidence suggesting that the major interaction between lipids and proteins in a membrane is hydrophobic, and a number of models for a membrane have been proposed on this basis (Benson, 1966; Lenard & Singer, 1966; Sjöstrand & Barajas, 1970; Vanderkooi & Green, 1970). Therefore organic solvents may provide a suitable medium for proteins that lie mainly within the hydrophobic region of a membrane. There are a few reports of organic solvents being used to dissolve membranes (Blumenfeld, 1968; Green, Haard, Lenaz & Silman, 1968; Lowden, Moscarello & Morecki, 1966; Rega, Weed, Reed, Berg & Rothstein, 1967; Schnaitman, 1969; Zahler & Wallach, 1967; Zwaal & van Deenen, 1968), but little advantage has been taken of this fact except to separate protein from phospholipid by gel filtration on

Sephadex LH-20. There have been, however, a number of studies of synthetic polypeptides in different organic solvents, with mainly optical rotatory dispersion being used to measure the α -helix content (Schellman & Schellman, 1964). In general the polypeptides exist in the α -helix configuration in such an environment. Addition of an organic solvent to a water-soluble protein also tends to stabilize the α -helices (Hermans, Puett & Acampora, 1969), so that proteins that are soluble in an organic solvent might be expected to possess considerable secondary structure.

Some progress, with the use of organic solvents, has been made in the study of membrane proteins (Curtis, 1969a,b). It was found that rat liver mitochondrial membrane proteins dissolved readily in acidified chloroform-methanol (2:1 v/v). As a first step in the study, it was necessary to be able to separate the different proteins of the membrane dissolved in the organic solvent. The technique of gel filtration was considered. Nyström & Sjörvall (1965) developed a procedure for the exhaustive methylation of Sephadex G-25 and G-50; the methylated Sephadex swelled in a number of organic solvents. With this material it was possible to separate phospholipids and steroids at least partly according to their size, though the chemical nature of the compound and the solvent affected the separation so that adsorption on the matrix played an important part. The methylation procedure has been applied to Sephadex G-75, G-100 and G-200, giving products that swell readily in chloroform, 2-chloroethanol and dimethylformamide as well as water, less readily in alcohols, and not at all in ether and acetone. Polystyrene fractions, each with a narrow range of molecular weight, were eluted from columns of methylated Sephadex G-75 and G-200 in the order of their molecular weights.

The gel filtration of mitochondrial membrane proteins was followed by polyacrylamide-gel electrophoresis with the buffer system of Neville (1967). Membrane protein dissolved in acidified chloroform-methanol (2:1 v/v) gave two separate peaks containing protein on a column of methylated Sephadex G-100 equilibrated with chloroform-methanol (2:1, v/v). On methylated Sephadex G-75 the slower peak merged with the faster, whereas on methylated Sephadex G-200 the peaks were separated slightly more than on methylated Sephadex G-100. The separation appeared to be mainly related to the size of the proteins. The equilibrating solvent was varied with the result that considerable differences in the separation with methylated Sephadex G-100 were observed. In the presence of 1 mM-hydrochloric acid in chloroform-methanol (2:1, v/v) the protein was eluted as a single peak at the void volume with some trailing protein. Polyacrylamide-gel electrophoresis showed

that the protein bands were somewhat separated within the single peak, suggesting that non-specific aggregation had not occurred. A similar result was obtained when the solvent contained 0.1 M-diethylammonium hydrochloride and 50 mM-acetic acid. In the presence of different concentrations of acetic acid the separation was the same as in its absence. The membrane protein also dissolved in chloroform-methanol (2:1, v/v) containing 1 M-diethylammonium hydrochloride and gave a single peak on methylated Sephadex G-100 equilibrated with chloroform-methanol (2:1, v/v) containing 1 M-diethylammonium hydrochloride, but on methylated Sephadex G-200 a much broader slower peak was obtained.

Membrane protein dissolved in acidified chloroform-methanol or as eluted from a column may be precipitated by addition of a base, by increasing the concentration of chloroform or by addition of ether. The precipitate readily redissolves in chloroform-methanol (2:1, v/v), though hydrochloric acid is required if base is used. The stepwise addition of ether results in a partial fractionation of the membrane proteins somewhat similar to that obtained by gel filtration.

Optical rotatory dispersion of total membrane protein in chloroform-methanol gave a value for b_0 that corresponds to 50% α -helix. This value is similar to that obtained for membranes dissolved in 2-chloroethanol (Lenard & Singer, 1966; Wallach & Zahler, 1966) and that for different membranes in aqueous suspension (Urry, Masotti & Krivacic, 1970).

The work was supported by grants from the Medical Research Council and the Cancer Research Campaign.

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Fractionation of the Proteins of Liver Plasma Membranes

By J. W. GURD, W. H. EVANS and H. R. PERKINS.
(National Institute for Medical Research, Mill Hill,
London N.W.7, U.K.)

Few analytical studies have been made on the plasma (surface)-membrane proteins of mammalian eukaryotic cells, owing mainly to difficulties in isolating sufficient amounts of pure fractions. The method of Neville (1960) for isolating liver plasma membranes was adapted to a low-speed rate-zonal separation through a sucrose density gradient, thus permitting the preparation of plasma membranes in larger quantities free from other subcellular organelles (Evans, 1970a). Polyacrylamide-gel electrophoresis of liver subcellular membrane fractions dissolved in aqueous sodium dodecyl sulphate has shown the presence of a wide range of proteins, and the plasma-membrane fraction has a characteristic profile distinct from that of intracellular membranes such as the smooth endoplasmic reticulum or mitochondrial membranes (Schnaitman, 1969; Evans, 1970b). The different protein pattern observed by gel electrophoresis is reflected in the different enzymic activities present. Liver plasma membranes are also characterized by the presence of large amounts of glycoprotein components, which are stained in polyacrylamide gels by the Schiff-periodate procedure (Evans, 1970b). Other features of the liver plasma-membrane fraction include the relatively high content of cholesterol and sphingomyelin (Patton, 1970), the presence of the specialized intracellular junctions such as tight junctions and desmosomes (Benedetti & Emmelot, 1968) and the presence of transplantation antigens (Evans & Bruning, 1970). In studying the proteins of the liver plasma membranes, we have devised fractionation procedures that exploit the different solubilities of the components in aqueous and organic solvents and in anionic detergents.

The proteins of mouse liver plasma membranes were fractionated by using the following stepwise procedure. Membranes that had been thoroughly

washed with iso-osmotic sodium chloride were extracted at 37°C for 1 h in 50 mM-carbonate-bicarbonate buffer-10 mM-EDTA, pH 9.8, and 20% of the membrane protein was solubilized. Polyacrylamide-gel electrophoresis in 0.1% sodium dodecyl sulphate showed that the soluble fraction consisted of 20 or more proteins of widely differing molecular weights. The soluble fraction was low in carbohydrate content, and staining of the gels with the periodate-Schiff reagent indicated only one major glycoprotein component. Suspension of the washed residue in cold aq. 33% (v/v) pyridine (Blumenfeld, 1968; Blumenfeld, Gallop, Howe & Lee, 1970) and centrifugation of the resulting mixture (170 000 g_{av} for 60 min) gave a clear supernatant containing 30–40% of the remaining protein, which was slightly enriched in sialic acid. Liver plasma membranes thus differ from erythrocyte membranes, in which the membrane sialic acid was found exclusively in the soluble fraction after fractionation by aqueous pyridine (Blumenfeld, 1968). Less protein was found to be soluble in pyridine when the initial alkaline extraction was omitted or when EDTA was not present during the alkaline treatment. Gel electrophoresis of the pyridine-soluble material showed the presence of 12–15 protein bands, a considerable simplification over the 25 or more bands found in the intact membrane. Further fractionation was obtained by dialysis of the pyridine-soluble fraction against 5% (w/v) sodium dodecyl sulphate-0.5 M-urea-30 mM-2-mercaptoethanol and gel filtration on Sepharose 6B in sodium dodecyl sulphate-urea-tris buffer, pH 8. Gel electrophoresis of pooled fractions showed that resolution on the basis of molecular weight occurred, but all fractions contained more than one protein component. The procedure described represents an appreciable advance towards the goal of resolving the liver plasma membrane into its constituent protein components and provides material for chemical and immunological analyses.

Suspension of washed mouse liver plasma membranes in 4% (w/v) sarcosyl (sodium lauryl sarcosinate)-2.3% (w/v) tris, pH 7.8, at 0°C resulted in the solubilization of 80% of the protein and 50–70% of the 5'-nucleotidase activity. The detergent-insoluble fraction was examined by electron microscopy and consisted mainly of amorphous material. However, unit membrane structure was visible in the region of the tight junctions and desmosomes. The sarcosyl-soluble proteins were fractionated on a sucrose density gradient [10–30% (w/v) sucrose dissolved in 0.5% (w/v) sarcosyl-tris, pH 7.8] by centrifuging at 95 000 g_{av} for 24 h (Beckman SW 27 rotor). Most of the phospholipids remained at the top of the gradient, while the proteins entered it. The protein peak sedimented in