BBA 75011

WATER-INSOLUBLE PROTEINS FROM SUBFRACTIONS OF SHEEP BRAIN MICROSOMES

K. GOT*, G. M. POLYA**, J. B. POLYA AND L. M. COCKERILL
Chemistry Department, University of Tasmania, Hobart, Tasmania (Australia)

(Received March 21st, 1966)
(Revised manuscript received September 5th, 1966)

SUMMARY

1. Microsomal subfractions were prepared from sheep brain and characterized by protein, RNA, and phospholipid content.

2. Colourless, water-insoluble protein from microsomal subfractions was solubilized by detergent and characterized by migration rates relative to bovine serum albumin through Sephadex columns. Particle weights in the range 90 000–300 000 were demonstrated for the various preparations.

3. *Crotalus adamanteus* venom depolymerized protein preparations from all subfractions and from total microsomes, yielding fractions characterized by low migration rates through Sephadex gel and corresponding to medium (40 000) and low (2000–12 500) particle weights. The depolymerizations are due to the action of phospholipase A.

4. The water-insoluble proteins from different microsomal subfractions were similar in size, solubility and depolymerization behaviour.

INTRODUCTION

Subcellular membrane structural proteins have been extracted by means of detergents from mitochondria[1–3], microsomes[3–5], chloroplasts[3,6,7] and erythrocyte stroma[3,8]. These protein preparations are similar in their relative non-polarity, insolubility in water, ability to bind phospholipid at neutral pH and ATP-binding properties[3,9].

Mitochondrial "structural protein" was isolated by GREEN and co-workers[2,3] and shown to bind cytochromes *a*, *b* and c_1, and cytochrome *c* in the presence of lipid[2,9–11], a variety of nucleotides, pyrophosphate and inorganic phosphate[12], mitochondrial phospholipids[13], synthetic alkylphosphates[9] and myoglobin[2].

* Present address: Department of Biochemistry, Medical School, University of Tasmania, Hobart (Australia).
** Present address: School of Biology, Flinders University, Adelaide, South Australia (Australia).

The readily polymerizing mitochondrial structural protein has a monomeric molecular weight of 22 000 (ref. 2) and has been thought responsible for the maintenance of the structural and functional integrity of mitochondrial membranes[3,9]. Many of the interactions of mitochondrial structural protein are regarded as expressions of hydrophobic bonding[14] due to a relatively high proportion of amino acids with non-polar side chains[2,9]. Mitochondrial structural protein is probably the chief site of amino acid incorporation in mitochondrial protein synthesis[15,16]. The location of structural protein in the scheme of mitochondrial ultrastructure has been reviewed[9,16–18].

WEINBACH AND GARBUS[19] have shown that mitochondrial structural protein binds some uncouplers of oxidative phosphorylation, the degree of binding being greater for more efficient uncoupling agents, and also demonstrated a monomeric molecular weight of 20 000. The identity of structural protein and the coupling factor F4 has been suggested[20,21]. Structural protein binds diphtheria toxin, which is an uncoupler of oxidative phosphorylation[22]. Water-insoluble proteins from erythrocyte stroma, microsomes and chloroplasts bind ATP and phospholipids[3].

The similarity of structural proteins from chloroplasts (monomeric mol.wt. 23 000) and mitochondria follows from interaction experiments[6]. Amino acid and ultracentrifugal analyses have been carried out on water-insoluble proteins extracted from chloroplasts from various sources[7,23–25]. Erythrocyte membrane protein has been extracted by means of detergents[3,26–28]. Water-insoluble protein from myelin lamellae has been completely or partly extracted using organic solvents[29,30] or lysolecithin[31] in which case a molecular weight of 28 000 has been estimated for the protein part of a myelin subunit.

Water-insoluble protein was isolated from sheep-brain microsomes and characterized by ultracentrifugal studies and interaction experiments[4]. RICHARDSON, HULTIN AND GREEN[3] showed that beef-liver microsomal structural protein was capable of binding lipid and ATP (ref. 3).

The purpose of this work has been to extract water-insoluble but detergent-soluble proteins from well-defined microsomal subfractions rather than from total microsomal preparations and to compare the proteins obtained from membranes of different kinds. In this way it was hoped to contribute to tests of the hypothesis that structural proteins from membranes of different cell organelles are identical or closely related.

METHODS AND MATERIALS

Sheep brain cell microsomes

Sheep brains, without standardisation of age or sex were kept over ice, and processing commenced within 1 h of obtaining them from the slaughterhouse. The brain was cleaned in ice-cold 0.25 M sucrose by removal of outer membranes and blood vessels, dried with blotting paper, weighed, cut up with scissors over ice and treated in a Waring blendor, after the addition of 0.25 M sucrose, to give a 20% pinkish homogenate. This was further treated in a Potter–Elvehjem homogeniser (all-round clearance 0.004 inch) and diluted with 0.25 M sucrose to give a 10% fine homogenate. The "mitochondrial" fraction was removed by 13 000 × *g* centrifugation for 10 min. Total microsomes were prepared from the 13 000 × *g* supernatant in the presence of CsCl (0.015 M) centrifuged at 200 000 × *g* for 75 min; the degree of

mitochondrial contamination was estimated by use of succinate dehydrogenase as a marker enzyme[32].

Subfractionation of sheep brain microsomes

The microsomes were subfractionated by the metal ion and discontinuous density-gradient centrifugation method of DALLNER[33]. Subfractions II (Cs^+ binding), Ia (Cs^+ and Mg^{2+} non-binding) and Ib (Cs^+ non-binding, Mg^{2+} binding) were prepared. The starting material for the subfractionations was the 13 000 × *g* supernatant itself or enriched with microsomal sediment.

Analytical methods

Succinate dehydrogenase activity was measured by the method of QUASTEL AND WHEATLEY[34] as modified by ALDRIDGE AND JOHNSON[35].

Protein measurements were carried out according to LOWRY *et al.*[36]. Using crystalline bovine serum albumin (Sigma Chemical Co.) as a standard, the solubilization procedure of ALDRIDGE AND JOHNSON[35] was used where necessary.

RNA determinations were made by the orcinol method or by 250.mμ readings on CLO_4 acid RNA extracts[37] or by the method of LITTLEFIELD *et al.*[38] employing an extinction coefficient of 34.2 mg^{-1} $ml \cdot cm^{-1}$. All three methods gave results in good agreement.

Phospholipid was measured by determining phospholipid P by the method of CHEN, TORIBARA AND WARNER[39], which involved an ethanol–ether (2:1, v/v) extraction of phospholipid; alternatively, the colorimetric method of the same authors was applied to phospholipids extracted by chloroform–methanol (2:1, v/v) (ref. 40). Results in terms of mg phospholipid P were converted to mg phospholipid by a correction factor of 25 (ref. 33).

Solubilization of membrane protein

The procedure followed was that of RICHARDSON, HULTIN AND FLEISCHER[13]. The method differed in some preparations. The subfractions to be extracted were treated with sodium cholate (1 mg/mg protein) and deoxycholate (2 mg/mg protein) and the mixture was stirred in the cold (1°) for about 15 min. In some cases 0.26% or 0.5% deoxycholate was used for the initial extraction. After centrifugation at high speed (50 000 × *g*–75 000 × *g*) for 25 min the supernatant was recovered, treated with 1/10 its volume of phosphate buffer (pH 7.0) and a few mg sodium dithionite and then stirred at 1° for 10 min to 12–16% saturation with $(NH_4)_2\,SO_4$. The precipitate was washed by taking it up in Tris–sucrose buffer (pH 9.0) and centrifuging. It was found necessary to wash this sediment further with 0.025 M Tris–HCl (pH 8.6) or 0.05 M phosphate buffer (pH 7.0) to remove sucrose and to prevent "stickiness" developing after the subsequent acetone extraction. The washed pellet was then extracted with 90% acetone in a salt–ice bath (−15°) for 15 min and the delipidized material recovered by centrifugation. The acetone-extracted pellet was allowed to dry at 1° and the protein was solubilized by 0.3% sodium dodecyl sulphate at pH 10.5, in many cases by successive extractions. Alternatively a mixture of 0.1% sodium dodecyl sulphate and 0.5 M urea was employed to solubilize the protein. Incubation at 25° was used to assist solubilization. The protein solutions were dialysed against 0.025 M Tris–HCl buffer in 0.05 M NaCl (pH 8.6) for not less than 16 h.

Sephadex chromatography

Sephadex G-100 and G-75 (Pharmacia, Uppsala) were used in columns of dimensions 50 cm × 2.5 cm and 50 cm × 20 cm. Procedures for chromatography followed those of ANDREWS[41] and FLODIN[42]. Elution volumes were determined by adding 2 ml of protein solution (<4 mg/ml) containing 10 mg sucrose, to the column, eluting with 0.025 M Tris–HCl buffer in 0.05 M NaCl (pH 8.6) and extrapolating the sides of the peaks obtained[41]. Alternatively, larger volumes (20-60 ml) of more dilute protein solutions were added to the column and elution volumes determined from the point of inflexion on the elution profile. Elution volumes were compared to those for crystalline bovine serum albumin to obtain values of R_{SA} = elution volume for bovine serum albumin/elution volume of protein. The data of ANDREWS[41] were used to determine particle weights, and the columns were calibrated with ovalbumin, human and bovine γ-globulin, bovine serum albumin and protamine sulphate. A Hilger-Watts 700 spectrophotometer was used to determine protein (measured at 260 mμ and 280 mμ) in the effluents, which were collected in 2- or 3-ml fractions.

Treatment with snake venom

As a general procedure, 1.8 ml of solubilized membrane protein containing approx. 3 mg protein were incubated at pH 8.9 for 3 h at 37° with preparations made from the venom of *Crotalus adamanteus*. After dilution to 10 ml with Tris–HCl buffer the solution was analyzed on Sephadex G-100 or G-75 columns.

In addition to untreated snake venom (1 mg) two kinds of treated material were used: 1 mg venom dissolved in 3 ml phosphate buffer (pH 5.9) was boiled on a water bath[43] for 5 min before incubation with membrane protein in the presence of Ca^{2+} ions (10^{-2} M) and the mixture was cleared by centrifugation at low speed before chromatography on Sephadex; alternatively phospholipase A was extracted from the venom by the method of CREMONA AND KEARNEY[44], 0.15 ml of the extract (with a protein content of 2.25 mg/ml) and Ca^{2+} ions (10^{-2} M) were incubated with the membrane protein.

RESULTS

Chemical analysis of microsomal subfractions from sheep brain

The protein, RNA and phospholipid distributions in subfractions II, Ia and Ib of sheep brain microsomes are given in Table I. The two preparations differ somewhat because of slight procedural differences but the overall distributions agree well with the data obtained by DALLNER[33] for corresponding rat-liver subfractions. Subfraction II contains the largest, and subfraction Ia the smallest amount of protein and RNA. Phospholipid is distributed more or less evenly between the different subfractions, but the phospholipid:protein ratio increases in the order II$\leqslant$Ib<Ia.

Sephadex G-100 chromatography of water-insoluble microsomal proteins

R_{SA} values for protein preparations from total microsomes and microsomal subfractions are given in Table II; the particle weights were estimated from the data of ANDREWS[41] and lie in the range of about 98 000–220 000. ANDREWS[45] has recently revised his data for the upper range of his V_e–log(mol.wt.) graph for proteins on Sephadex G-100; thus our particle weights may require an upward correction.

TABLE I

DISTRIBUTION OF PROTEIN, RNA AND PHOSPHOLIPID IN CELL FRACTIONS OF SHEEP BRAIN

Fractions Ia, Ib and II represent Mg^{2+} non-binding, Mg^{2+} binding and Cs^{+} binding subfractions respectively. Succinate dehydrogenase activity of the 13 000 × *g* sediments was in the range of 38–50 μl CO_2 per min per g brain and that of the 13 000 × *g* supernatants 4.3–4.9 μl CO_2 per min per g brain.

Fractions	*Expt. No.*	*Protein (mg/g brain)*	*RNA (mg/g brain)*	*RNA : protein*	*Phospholipid (mg/g brain)*	*Phospholipid: protein*
Homogenate	1	120.0			45.2	0.38
	2	90.0			52.6	0.57
13 000 × *g* sediment	1	69.3			41.1	0.59
	2	50.0			....	....
13 000 × *g* supernatant	1	29.3	0.721	0.025	2.60	0.09
	2	17.5	0.805	0.046	4.40	0.25
Total microsomes	1	20.0	0.628	0.032	2.45	0.12
	2	9.7	0.372	0.038	3.03	0.31
Supernatant	1	10.0	0.196	0.020	0.95	0.09
	2	5.8	0.203	0.035	0.37	0.06
Recovery Step I	1	83%			98%	
	2	74%			...	
Ia	1	1.5	0.040	0.027	0.40	0.26
	2	1.3	0.020	0.016	0.73	0.56
Ib	1	2.3	0.073	0.032	0.23	0.10
	2	3.0	0.073	0.025	1.04	0.35
II	1	4.1	0.261	0.079	0.45	0.11
	2	3.0	0.330	0.110	0.44	0.15
Supernatant after metal ion fractionation	1	13.9	0.200	0.014	1.88	0.14
	2	4.7	0.123	0.026	0.52	0.11
Loss in conc. sucrose layers	1	1.9	0.07		—	
	2	—	—		—	
Recovery from 13 000 × *g* supernatant	1	79%	78%		87%	
	2	69%	76%		80%	

TABLE II

PARTICLE WEIGHTS OF PROTEIN PREPARATIONS DETERMINED FOR SEPHADEX G-100 AND ULTRACENTRIFUGALLY

Source	R_{SA}	*Sephadex*	*Ultracentrifuge*
Ia	1.26	141 000	90 000–180 000
	1.20*	120 000	
Ib	—	>100 000	230 000–280 000
Microsomes	1.14	98 000	<137 000
"Reticulosome"	1.20	119 000	—
	0.57	9 800	
II	1.39	219 000	174 000
	0.456	3 800	

* Dilute protein solution in large volume placed on column.

Ultracentrifugal studies on these preparations were rendered inaccurate by the low protein concentrations, uncertainty of the degree of detergent binding to the protein, possible denaturation of the protein and introduction of undesirable change effects due to detergent binding and probable polydispersity of the systems, but they indicate particle weights of the same order, in the range of 90 000 to 280 000.

Snake venom treatment of protein preparations

Partial but far going depolymerisations were demonstrated for total microsomes and subfractions Ia, Ib and II; particle weights of the small components isolated after treatment with unprocessed snake venom were in the range of 2000 to 12 500. Elution profiles of selected preparations from total microsomes and subfractions II, Ia and Ib untreated and treated with unprocessed snake venom are shown in Figs. 1, 3–6. In preparations of subfraction II (Figs. 5 and 6) components of small particle weight are evident before treatment with snake venom but treatment increases the quantitative significance of these peaks.

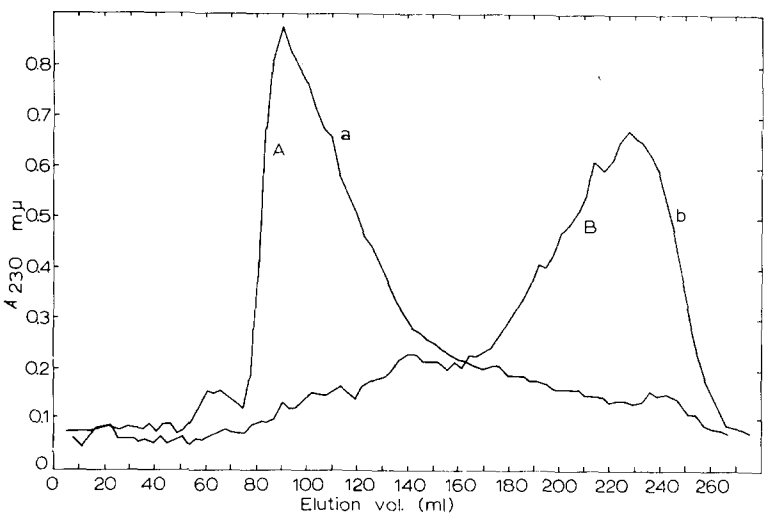

Fig. 1. Sephadex-gel filtration (G-100) of solubilised proteins from total microsomes. 0.025 M Tris–HCl in 0.05 M NaCl (pH 8.6). a, untreated; b, after treatment with snake venom. A, R_{SA} = 1.14; particle weight = 98 000. B, R_{SA} = 0.45; particle weight = 3500–3600.

Fig. 2 compares the effects of unprocessed, boiled and extracted snake venom on total microsomes. Broadly speaking the three elution profiles are similar. In the region of low particle weights the maximum corresponds to about 12 500. The phospholipase A extract gives a higher yield of products of low particle weight but the sharp band corresponding to particle weight 40 000, which is clearly visible on the two other curves, is missing or barely indicated in this case. Similar results have been obtained on fraction Ib.

The trailing edges of the profiles of untreated preparations and the advancing edges of the profiles of the treated preparations indicate thet heterogeneity which is

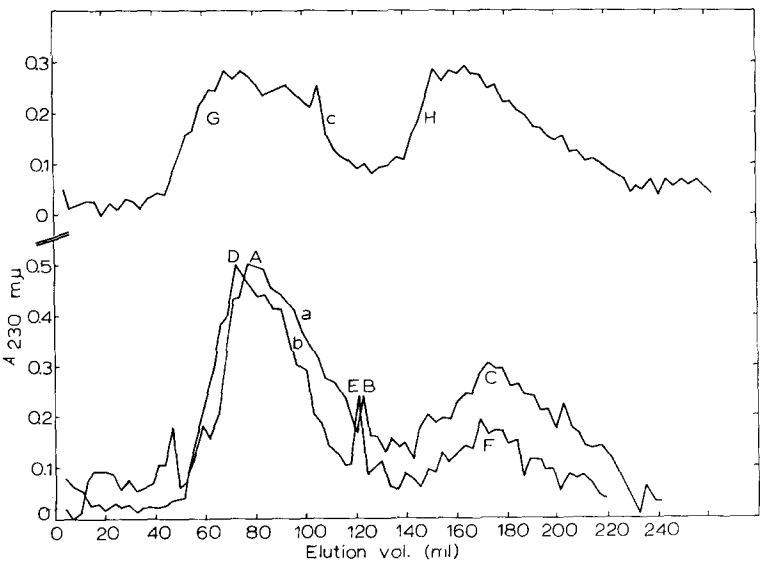

Fig. 2. Sephadex-gel filtration (G-100) of solubilised protein from total microsomes. 0.025 M Tris–HCl in 0.05 M NaCl (pH 6.6). a, treatment with unprocessed snake venom; b, treatment with boiled snake venom; c, treatment with phospholipase A extracted from snake venom. For untreated protein, see Fig. 1. A, —; particle weight > 100 000. B, R_{SA} = 0.76; particle weight = 40 000. C, R_{SA} = 0.57–0.48; particle weight = 5000–12 500. D, — ;particle weight > 100 000. E, R_{SA} = 0.76; particle weight = 40 000. F, R_{SA} = 0.57–0.48; particle weight = 5000–12 500. G, —; particle weight > 100 000. H, R_{SA} = 0.6–0.48; particle weight = 5000–20 000.

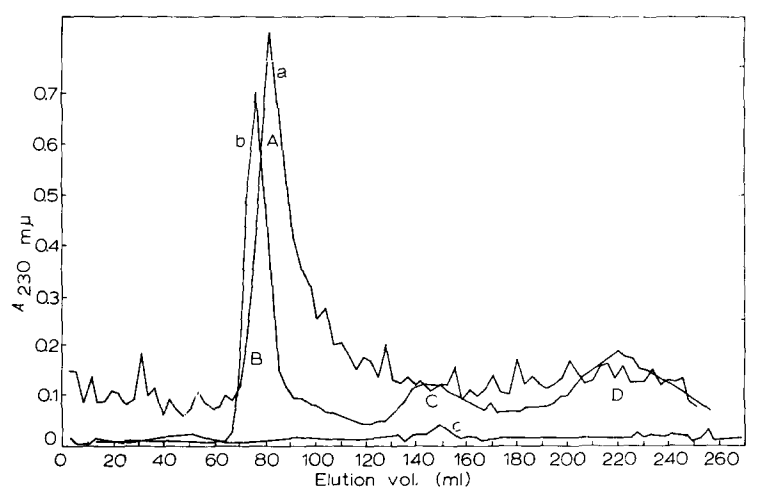

Fig. 3. Sephadex-gel filtration (G-100) of solubilised protein prepared from Mg^{2+} non-binding subfraction. 0.025 M Tris–HCl in 0.05 M NaCl (pH 8.6). a, untreated; b, after treatment with snake venom; c, snake venom. A, R_{SA} = 1.26; particle weight = 141 000. B, R_{SA} = 1.43; particle weight = 260 000. C, R_{SA} = 0.74; particle weight = 30 000. D, R_{SA} = 0.49; particle weight = 5500.

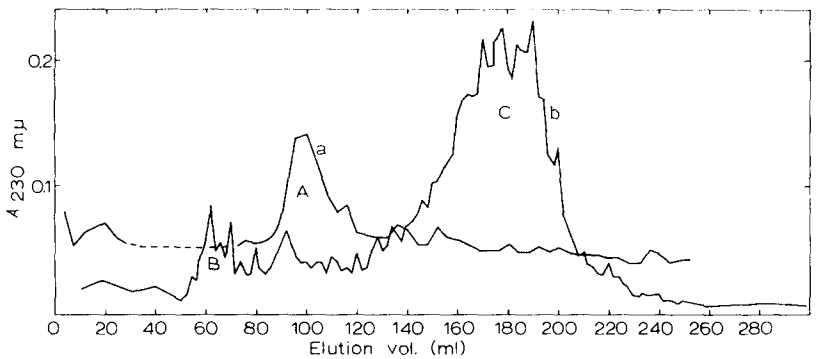

Fig. 4. Sephadex-gel filtration (a, G-100; b, G-75) of solubilised protein prepared from Mg^{2+} binding subfraction. 0.025 M Tris–HCl in 0.05 M NaCl (pH 8.6). a, untreated; b, after snake venom treatment. A, —; particle weight > 100 000. B, R_{SA} = 1.09; particle weight = 110 000. C, R_{SA} = 0.34–0.40; particle weight = 1800–4300.

also evident from ultracentrifugal results (although single major elution peaks were obtained in most preparations and single schlieren peaks were observed ultracentrifugally).

Analysis of a "reticulosome" subfraction

Subfraction II material which had been treated successively with 0.26 or 0.5% deoxycholate and ribonuclease yielded fractions with a phospholipid:protein ratio of 0.07 and RNA:protein ratio of 0.02, closely similar to the findings of POLLAK AND SHOREY[46] on reticulosome preparations from embryonic chick liver: RNA:protein<

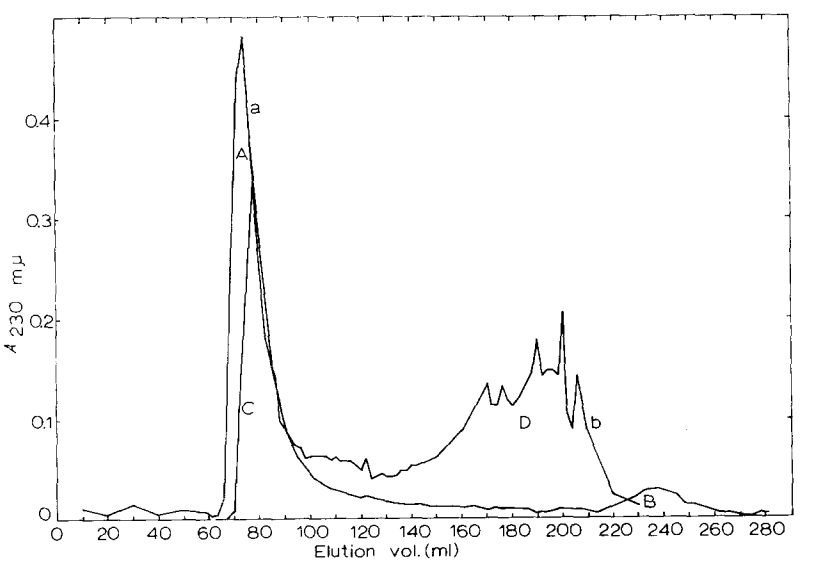

Fig. 5. Sephadex-gel filtration (a, G-100; b, G-75) of solubilised protein prepared from Cs^{+} binding subfraction. 0.025 M Tris–HCl in 0.05 M NaCl (pH 8.6). a, untreated; b, after snake venom treatment. A, R_{SA} = 1.39; particle weight = 219 000. B, R_{SA} = 0.46; particle weight = 3800. C, R_{SA} = 1.09; particle weight = 110 000. D, R_{SA} = 0.43; particle weight = 5000.

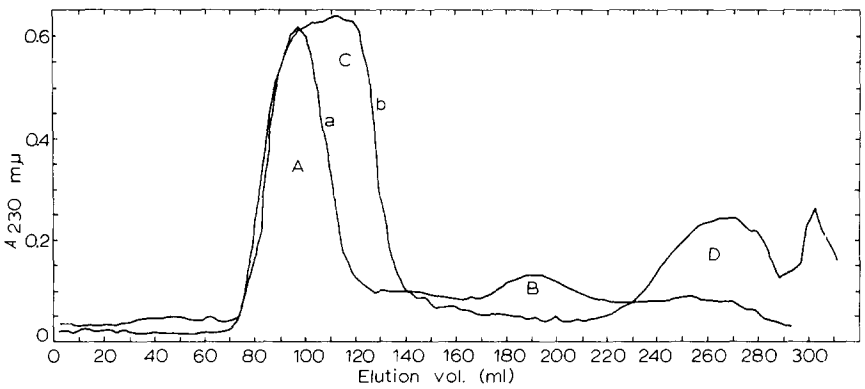

Fig. 6. Sephadex-gel filtration (G-100) of solubilised protein from "reticulosomes". 0.025 M Tris–HCl in 0.05 M NaCl (pH 8.6). a, untreated; b, after snake venom treatment. A, R_{SA} = 1.20; particle weight = 119 000. B, R_{SA} = 0.57; particle weight = 9800. C, R_{SA} = 1.23; particle weight = 129 000. D, R_{SA} = 0.43; particle weight = 3000.

0.04; phospholipid:protein 0.08. Water-insoluble protein extracted from this fraction had a particle weight of 119 000 for the major fraction as well as a minor fraction of particle weight 9800 (Table II). *C. adamanteus* venom depolymerized this preparation to a particle weight of 4500 (Fig. 5).

DISCUSSION

The protein RNA and phospholipid distributions are in agreement with DALLNER's findings[33] and thus should correspond to the following morphological assignments demonstrated electron-microscopically for analogous subfractions of rat-liver microsomes: subfraction II: rough vesicles and ribosomes; subfraction Ib: smooth vesicles deriving from the endoplasmic reticulum; subfraction Ia: smooth vesicles of a different nature originating possibly from the glycogen area of cytoplasm but not from the cytoplasmic reticulum. The water-insoluble proteins extracted from total microsomes and from the "rough-vesicle" (II) and "smooth-vesicle" (Ia and Ib) subfractions were similar in that they were colourless and water-insoluble, but detergent-soluble. No significant haemoprotein contamination was detected spectroscopically. These properties are similar to those of the structural protein prepared from mitochondrial membranes by GREEN and co-workers[1,2] who have proposed that the element of universality in all membrane systems may be a molecular network of structural protein and lipid[14].

The usual particle weights of the preparations from all subfractions lie in the range of 100 000–300 000 and all behave similarly when treated with the venom of *C. adamanteus*, which is a rich source of phospholipase A (refs. 43, 47). Since both the extract made by the method of CREMONA AND KEARNEY[44] and boiled venom give practically the same results as the unprocessed venom, the depolymerisations observed must be ascribed to the action of phospholipase A and to proteinases which are inactivated by boiling[43].

This conclusion is attested by other, indirect, proofs. Even untreated *C. adamanteus* venom had no depolymerizing action upon bovine serum albumin: chromato-

graphy on Sephadex G-75 after treatment gave a peak with $R_{SA} = 0.99$ as compared with $R_{SA} = 1.00$ of the untreated sample; minor, slower peaks were not observed after treatment. If the depolymerizations were due to snake venom proteinase, one would expect a protein preparation subjected to successive treatments with 0.1% sodium dodecyl sulphate and 0.5 M urea to be depolymerized more readily than protein preparations merely extracted with one treatment of 0.3% of the detergent. However the former preparation was found to be little affected by snake venom treatment while preparations of the latter type were drastically depolymerized, although proteinases would be expected to act more effectively upon proteins subjected for further denaturation by urea treatment.

The clearing action of lysolecithin on brain homogenate[48] and the requirement of phospholipid for organisation into vesicles[49] may be relevant to the interpretation of the depolymerization under discussion.

Molecular weight estimates are 22 000 (refs. 1,2) and 20 000 (ref. 19) for mitochondrial, 23 000 (ref. 6) for chloroplast and 28 000 for myelin[31] membrane "structural protein" subunits. Estimates for the molecular weights of the subunit of microsomal "structural protein" range from 15 000 to 30 000 (ref. 4). Since the fractions of low particle weight have been obtained by the action of phospholipase A and not by proteinases, the hypothesis of sub-units smaller than those suggested before must be considered. The repeated observation of particle weights of 40 000 with moderately active enzyme preparations is in good agreement with postulates of sub-units of the order of 20 000. Sub-units of particle weights in the range of 5000–12 500 provide plausible largest common divisors of the values quoted above. An acid-soluble protein fraction from myelin with a particle weight of 10 000 (ref. 50) is interesting to note in this connection.

All of the subfractions prepared, including the "reticulosome" fraction yielded water-insoluble proteins of similar particle size which were depolymerized by snake venom treatment to sub-units which were again similar. This argues for a basic similarity of the different types of membrane systems of the microsomal fractions. The "reticulosomes" prepared by POLLAK AND SHOREY[46] consisted of lipoprotein granules about 150 Å diameter, and POLLAK[51] has shown that reticulosomes can combine with micellar phospholipid to give rise to typical membrane structures that behave like microsomal smooth membranes in discontinuous density-gradient centrifugation. Recent work by MANGANIELLO AND PHILLIPS[52] has demonstrated great similarity between the rough and smooth membrane fractions from liver microsomes, especially with regard to the appearance of new protein in the membranes.

The findings reported here provide further evidence for the fundamental similarity between different cytomembranes but it has to be supplemented by interaction experiments that are now in course of investigation.

ACKNOWLEDGEMENTS

One of us (G.M.P.) gratefully acknowledges the Mason Memorial Scholarship to the Royal Australian Chemical Institute. The authors are indebted to Professor D. E. GREEN and Dr. J. K. POLLAK for helpful discussion.

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