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A PURIFIED PLASMA MEMBRANE FRACTION ISOLATED FROM RAT LIVER UNDER ISOTONIC CONDITIONS

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SUMMARY

A fraction rich in plasma membrane has been isolated from perfused rat liver. The purity of the preparation is comparable with that of material isolated by other procedures and the yield of the purified fraction is appreciably greater. Isotonic sucrose is used in the early stages of the cell fractionation and it is therefore possible to isolate both a plasma membrane fraction and other cell components from the same homogenate.

INTRODUCTION

5'-Nucleotidase is probably confined in the liver cell to the plasma membrane[1,2]. In subcellular fractionations of liver this enzyme usually appears mainly in nuclear, lysosomal, and microsomal pellets, but the distribution between these pellets is very variable[2-7,**].

It seemed that these variations reflected differences in distribution of the fragmented plasma membrane, probably caused by the varied homogenization conditions used by different workers. This observation has now been used as the starting point for the preparation described here. A 'nuclear' pellet, containing much of the nucleotidase of the homogenate (50–70%), is prepared from gently homogenized perfused livers. After vigorous homogenization of this pellet, the nucleotidase can mostly be washed out of the nuclear pellet and is sedimented only at higher speeds. The partially purified material thus isolated can then be further purified by density-gradient centrifugation.

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** R. H. MICHELL AND R. COLEMAN, unpublished results.

METHODS

Protein was determined by a biuret method, modified so as to avoid interference by sucrose and by lipids[8].

Total nucleic acids[9], deoxyribonucleic acid[10] and ribonucleic acid[11], were estimated on trichloroacetic acid extracts of the fractions[11].

Lipids were extracted from each fraction by mixing 1 vol. with 3.75 vol. of chloroform–methanol (1:2, v/v) for 15 min at room temperature. 1.25 vol. of chloroform were then added followed by 1.25 vol. of 0.2 M HCl. The lower phase was then washed with top phase containing 0.1 M $MgCl_2$, dried down *in vacuo* and the resulting extract dissolved in 10 ml chloroform. Aliquots of this extract were used for the determination of phospholipid as total phosphate[12], and cholesterol[13,14].

Enzymatic determinations

The following activities were measured: succinate dehydrogenase, EC 1.3.99.1 (as succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase) (ref. 15); glucose-6-phosphatase, EC 3.1.3.9 (ref. 16); 6-phosphogluconate dehydrogenase (decarboxylating), EC 1.1.1.44 (ref. 17); uricase, EC 1.7.3.3 (ref. 18); 5′-nucleotidase, EC 3.1.3.5 (ref. 7); Mg^{2+}-ATPase, EC 3.6.1.3 (ref. 19).

Acid phosphatase, EC 3.1.3.1 (ref. 20), and β-glucuronidase, EC 3.2.1.31 (ref. 21), were assayed after the use of a procedure for the activation of structure-latent enzymes[20].

Preparation of fractions for electron microscopy

The method of COLEMAN AND FINEAN[22] was used.

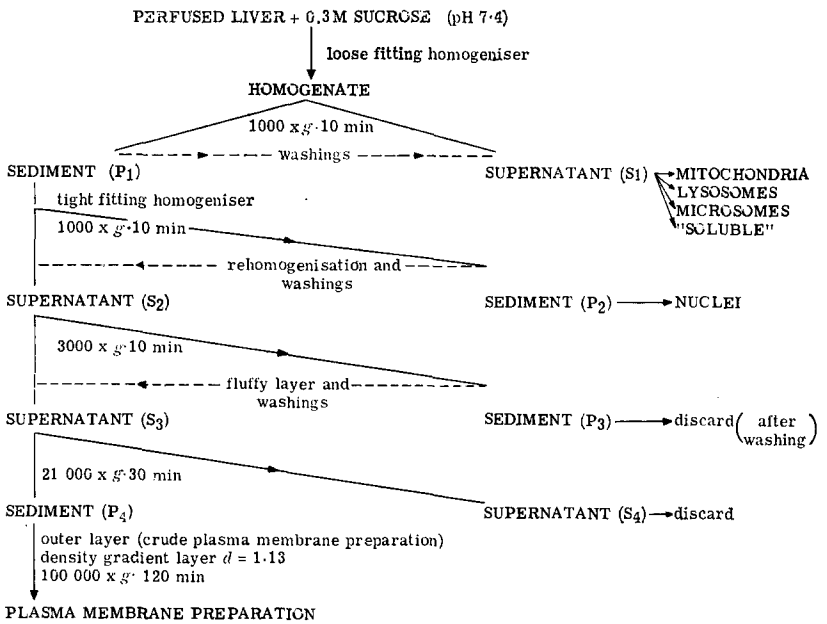
RESULTS

Isolation of the purified membrane fraction

Scheme I represents an outline of the procedure. Livers of 6–8-week-old rats of either sex, under ether anaesthesia, were perfused *in situ* with warmed (37°) 0.3 M sucrose (pH 7.4) or 0.3 M sucrose–2 mM $MgCl_2$–2 mM $CaCl_2$ (pH 7.4) (in some experiments) *via* the hepatic artery. The livers were then removed and quickly chilled. All subsequent operations were performed at 0–4°. The livers were homogenized in 0.3 M sucrose (adjusted to pH 7.4 with $NaHCO_3$) using a Teflon–glass Potter–Elvehjem homogenizer (pestle diameter 18.65 mm, radial clearance 0.39 mm) at 500 rev./min using 6 complete strokes. 0.3 M sucrose (pH 7.4) (which was used in all subsequent operations) was used to adjust the volume to 8 ml/g of tissue.

The homogenate was centrifuged at 1000 × *g* for 10 min. The resulting pellet was washed 3 times by homogenization as above, but in a volume half that of the original homogenate, the pellet being sedimented at 1000 × *g* for 10 min on each occasion. The combined supernatants from these spins (S_1) were suitable for the preparation of further subcellular fractions (mitochondria, lysosomal, microsomal and soluble) by standard techniques (*e.g.* ref. 23).

The thrice-washed pellet (P_1) was suspended in a volume three-eighths that of the original homogenate and was homogenized more vigorously (Teflon–glass Potter–Elvehjem homogenizer, pestle diameter 24.53 mm, radial clearance 0.20 mm, 2000



SCHEME I. Summary of fractionation

rev./min, 20 complete strokes). A pellet was sedimented at 1000 × *g* for 10 min and the supernatant retained (S_2). The pellet was twice rehomogenized vigorously and sedimented in the same volume. The pellet remaining consisted largely of nuclei with intact nuclear membranes.

The three supernatants were combined and a pellet sedimented at 3000 × *g* for 10 min. The supernatant and the loose pink layer at the outer surface of the pellet (P_3) were decanted and combined with the previous supernatant. These supernatants (S_3) were then centrifuged at 21 000 × *g* for 30 min. The pellets, including the loosely packed surface layer, were suspended in one-fifth the volume of the original homogenate and sedimented at 21 000 × *g* for 30 min.

The resulting pellet (P_4) was triple layered, consisting of a small button of mitochondria overlaid by a firm, then a loose pinkish-white layer. Most of the supernatant (S_4) was decanted, care being taken not to lose any of the loose fluffy material. The loose layer was then resuspended in a total volume of 40 ml or less, by swirling the tubes by hand and decanting the resulting suspension. This suspension, consisting of a partially purified plasma membrane fraction was split into four equal portions. One of these was kept for analysis (relative specific activity of 5'-nucleotidase compared to homogenate is usually in the region of 8 at this stage), and the other three were loaded onto discontinuous gradients of sucrose of the following composition: density 1.18–2 ml, density 1.16–3 ml, density 1.13–5 ml. The tubes were centrifuged for 2 h at 100 000 × *g* in the 3 × 20 ml swing-out head of an MSE "50" centrifuge.

A major pinkish-white band was seen at the top of the density 1.13 layer, a lesser one at the 1.13/1.16 interface and a pink pellet at the bottom of the tube. The material at the top of the density 1.13 layer was collected, diluted slowly with water to approx. 0.3 M in sucrose and stored at −20° as the final purified plasma membrane preparation.

Yield of membranes

Three preparations are documented in Table I. The yields of these were: Prep. 1: 62 mg protein (16.9-fold purification of 5'-nucleotidase) from 60 g liver; Prep. 2: 92 mg protein (13.1-fold purification) from 40 g liver; Prep. 3: 48 mg (23.6-fold purification) from 55 g liver.

TABLE I

ENZYMATIC AND CHEMICAL CONTENT OF PLASMA MEMBRANE PREPARATIONS

n.d. stands for not determined. Relative specific activity = specific activity of preparation: specific activity of original homogenate. INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium.

	Specific activity			*Relative specific activity*			*Yield (% of homogenate)*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Protein	—	—	—	—	—	—	0.7	1.3	0.6
5'-Nucleotidase (plasma membrane)	31.3*	52.5	91.0	16.9	13.1	23.6	11.2	17.8	14.1
Mg^{2+}-ATPase	21.2*	8.5	39.3	7.9	3.7	9.5	4.7	4.8	5.7
6-Phosphogluconate dehydrogenase (soluble)	0**	0	0	0	0	0	0	0	0
Succinate-INT reductase (mitochondria)	0.022*	0.15	0	0.017	0.17	0	0.012	0.22	0
Acid phosphatase (lysosomes)	0	0.074	0	0	0.39	0	0	0.51	0
β-Glucuronidase	0.027*	0.061	0.035	0.5	1.07	0.54	0.26	1.37	0.32
Uricase (peroxisomes)	0.39*	0.18	0.29	1.14	0.64	1.05	0.8	0.96	0.63
Glucose-6-phosphatase (endoplasmic reticulum)	1.47*	0.78	6.9	0.56	0.23	2.05	0.17	0.07	1.02
RNA (principally endoplasmic reticulum)	2.5†	7.4	7.1	0.55	1.96	1.42	0.37	2.5	0.86
DNA (nuclei)	0.83†	4.0	3.9	0.57	2.4	2.0	0.39	3.2	1.19
Total nucleic acid	n.d.	n.d.	n.d.	0.63†	1.82	0.82	0.4	1.82	0.82

* μmoles substrate utilized or product liberated per h per mg protein at 37°.
** μmoles substrate utilized per h per mg protein at 25°.
† μmoles nucleic acid P per mg protein.

Characteristics of the preparation

Samples of the preparations taken for electron microscopy at the purified stage contained collections of vesicles and strips of membrane of many sizes. Occasionally a desmosome, a feature of the plasma membrane in the tissue, was observed. A small proportion of the sample contained membranes bearing granules, suggestive of ribosomes, which might be expected from the RNA content of the fraction. The various sizes of the vesicles indicated that the second homogenization step had caused excessive fragmentation of the plasma membrane. Fragmentation of outer mitochondrial and nuclear membranes under these conditions had been much less extensive, since these organelles could be isolated from prior fractions to the plasma membrane with their membranes intact, even after the second homogenization step.

Enzymatic activities and purity of the final preparations

Three consecutive preparations are documented in Table I, which, although showing varying degrees of purity, demonstrate a very considerable purification away from other cellular material. Table I lists the activities of a variety of enzymes and the nucleic acid contents of each preparation. The analyses included are those generally accepted as biochemically typical of single cell components and therefore reflect the levels of those cell components present in the preparations. These structures are given in parentheses against each analysis.

It is clear that in each preparation the plasma membrane (exemplified by 5′-nucleotidase levels) was relatively free of other cell components. Considerable concentration of Mg^{2+}-ATPase was also achieved, but in this case the extent of purification was lower due to Mg^{2+}-ATPase activity in other cell components, notably mitochondria.

In spite of the considerable enrichment with respect to plasma membrane, all of the preparations showed appreciable though variable contamination with nuclear (DNA) peroxisomal (uricase) and endoplasmic reticulum (glucose-6-phosphatase and RNA) material. Mitochondrial and lysosomal contamination was very slight and 'soluble' components (represented by the level of 6-phosphogluconate dehydrogenase) were not detectable. In each case the activities of all material derived from the original homogenate were assayed and recoveries close to 100% were obtained.

TABLE II

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF PLASMA MEMBRANE PREPARATIONS

Prep. No.	*mg cholesterol / mg protein*	*mg phospholipid* / mg protein*	*mg total lipid / mg protein*	*Molecular ratio cholesterol to phospholipid*
1	0.13	0.515	0.645	0.49
2	0.107	0.42	0.527	0.49
3	0.23	0.75	0.97	0.60

* μg phospholipid P × 25.

Lipid content

The preparations were rich in lipid. Table II gives the cholesterol and phospholipid content of the total lipid extracts made from the preparations. The weight of total lipid present (as cholesterol and phospholipid) was almost equal to that of protein in the most highly purified specimen.

DISCUSSION

A variety of marker criteria indicated that contamination of the preparation with other subcellular structures was similar to that observed by EMMELOT *et al.*[19], who studied membrane isolated mainly in hypotonic bicarbonate solution by a variant of the method of NEVILLE[24]. Purification, assessed as the specific activity of 5′-nucleotidase in the preparation, is approximately the same as, or occasionally greater than that reported by EMMELOT *et al.*[19]. Since the specific activity of the

final preparation is dependent not only on the relative purification but also upon the specific activity of the original homogenate, the specific activity can only be used as a rough guide to purity. The purified material is also similar to material obtained as Peak 3 of a zonal centrifugal resolution of the crude nuclear fraction of liver prepared in 0.08 M sucrose[25].

Initial experiments in the present study were carried out with non-perfused liver and it was found that partially purified membranes (P_4) were then reddish-brown in colour. Spectral analysis showed that the colour was largely due to the presence of haemoglobin. Perfusion of the liver prior to homogenization greatly reduced the colour of the final preparation. Perfusions were therefore carried out routinely in order to: (i) minimise contamination from erythrocyte plasma membrane; (ii) minimise the possibility of adsorption of haemoglobin and other blood proteins. Contamination with erythrocyte membranes should be avoided if possible, since the chemical and enzymatic properties of this species of plasma membrane might otherwise modify the properties of the isolated, supposedly purified, liver plasma membrane.

The observed difference in density between plasma membrane prepared by the present method and by that of EMMELOT *et al.*[19] is also reflected in the higher lipid/protein ratio of the material described here. It is generally accepted that the more protein a particulate system contains the greater will be its density. Two explanations for the altered ratio are possible: (i) the lower amount of protein in the present preparation represents a loss of protein during preparation (*e.g.* at the second homogenization step) or (ii) that some of the extra protein in the preparation of EMMELOT *et al.*[19] represents protein adsorbed during their preparative procedures. EMMELOT AND BOS[26] have demonstrated that triosephosphate dehydrogenase is adsorbed during their preparative procedures and that this can be eluted from the membrane with other saline soluble proteins. They have suggested[26] that at least part of these saline soluble proteins have been adsorbed to the membrane during the homogenization and dilution stages of their preparation, and in view of this we favour the second of the above explanations for the difference in the respective lipid/protein ratios. The material described in the present communication may therefore resemble more closely the saline extracted preparation of EMMELOT *et al.*[19], and it is also possible that some of the coloured protein, found to be saline soluble by EMMELOT *et al.*[19], is related to the haemoglobin which was present in our own non-perfused preparations.

Since purified samples of other cellular components are obtainable from the original homogenate, it is hoped that the present technique, in addition to providing a source of plasma membrane, will help in biosynthetic and other studies where a detailed subcellular distribution is required from each sample of liver.

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