Biochimica et Biophysica Acta, 352 (1974) 52–63

BBA 76639

STRUCTURAL STUDIES OF A MITOCHONDRIA-FREE PLASMA MEMBRANE FRACTION FROM RAT LIVER*

NICHOLAS WONG and JAMES E. ZULL**

Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106 (U.S.A.)

(Received December 14th, 1973)

SUMMARY

Liver plasma membranes virtually free of contaminating mitochondria have been prepared. Sodium dodecylsulfate–polyacrylamide gel electrophoresis reveals a membrane protein resistant to papain digestion in the intact membranes but readily hydrolyzed in membranes disrupted by detergent or sonication.

Electron microscopy of mechanically deformed membranes reveals fibrils within the membrane which appear to be protein in nature but which also persist in papain digested membranes.

INTRODUCTION

The nature of plasma membrane proteins and their molecular orientation in the membrane has been a subject for intensive research during the last five years [1–3]. A variety of techniques has been utilized for these studies, one of the more powerful of which is disc-gel electrophoresis in sodium dodecylsulfate [4–6]. Analysis by this technique has led to the characterization of certain patterns for certain membrane types, and studies on the susceptibility of specific membrane protein to various labeling procedures [7–11] or proteolytic enzymes [12, 2] has allowed some conclusions concerning the likely localization of specific proteins within the membrane. For example, Bretscher has concluded that a major protein, Component a, of the erythrocyte membrane extends completely across the membrane since it can be labeled from both sides of the membrane. Further, support for this conclusion is drawn from the work of Bender et al. [12] who showed that Component a in intact cells is cleaved by pronase while most other proteins are not, but that all proteins are susceptible to digestion in erythrocyte ghosts.

However, in studying the molecular architecture of plasma membranes from tissues other than blood, one of the major problems is the preparation of a fraction which does not contain major amounts of contaminating fragments from other subcellular locations, e.g. mitochondrial and endoplasmic reticulum. Clearly, in disc-gel

* This work is described in more detail in the Ph. D. thesis of N. O. Wong, 1972.

** To whom correspondence should be sent.

analysis of membrane proteins from preparations which contain 5% mitochondrial contamination, a major mitochondrial protein may be interpreted to be a minor, but significant membrane protein. Since most of the plasma membrane preparations reported in the literature contain at least 5% mitochondrial contamination and up to 15% endoplasmic reticulum [13–19], this is a significant problem in the field.

The data in this paper describe a method for preparation of plasma membranes from rat liver which contains virtually no detectable mitochondrial contamination, and about 7% endoplasmic reticulum. This preparation has been studied both in regard to the membrane protein response to proteolytic digestion and the existence of protein "fibrils" in the membrane as revealed by deformation–electron microscopy techniques.

METHODS AND MATERIALS

Membrane preparation

White, female Sprague–Dawley rats of about 150 g body weight were used. They were starved for 24 h prior to use. The animals were stunned and decapitated and the blood was drained. Liver was removed and placed in Medium I (unbuffered 250 mM sucrose, 3 mM $CaCl_2$) over ice. The liver was minced to about 1-mm cubes, and washed repeatedly in Medium I to remove excess blood. Then the liver was weighed and made into a 10% (w/w) mixture with Medium I. These and subsequent steps were all carried out over ice. The 10% (w/w) liver and Medium I mixture was homogenized in a 30-ml Thomas teflon pestle homogenizer No. 60 motor at a speed of about 2 rev./s. Four up-and-down strokes were employed. The homogenate was diluted with an equal volume (1 vol.) of Medium I and filtered through two layers of cheesecloth. The mixture was centrifuged at $600 \times g$ for 10 min.

The pellet thus obtained was gently rehomogenized in 1 vol. of Medium II (80 mM sucrose, 5 mM Tris, pH 7.6) with five up-and-down strokes. The homogenization was performed gently. The homogenate was diluted with 1 vol. of Medium II and centrifuged at $1000 \times g$ for 10 min.

At the end of this centrifugation step, the homogenate was separated into three layers, a solid reddish-brown pellet at the bottom, covered by a light, off-white fluffy layer, and the supernatant. The supernatant was decanted and discarded, after which the fluffy layer was also decanted and collected. The reddish-brown pellet was rehomogenized twice, and each time the fluffy layer was decanted and combined with that previously collected. Finally, the fluffy layer was washed twice, each time with 1 vol. of Medium II by resuspension and centrifugation at $1000 \times g$ for 10 min.

The pellet was resuspended in Medium II so that material from 5 g of liver was contained in 3.1 ml. To this, 3.9 ml of 69% sucrose–EDTA (3 mM) solution of density 1.35 was added and thoroughly mixed to a final density of 1.22. On top of this, 8.9 ml of sucrose solution of density 1.18 was layered followed by 5 ml of sucrose solution of density 1.16. The density gradient was centrifuged at $100\,000 \times g$ for 75 min. The material at the interface of the $d = 1.16$ and $d = 1.18$ layers was collected, and washed twice by suspending in 1 vol. of 1 mM Tris, pH 7.6 and centrifuging at $1000 \times g$ for 10 min. This procedure sediments essentially all of the plasma membrane fraction. The final pellet was stored in a small volume of 1 mM Tris, pH 7.9 at 4 °C.

The supernatant obtained after the $600 \times g$ centrifugation step from the original homogenate was centrifuged at $10\,000 \times g$ for 10 min to sediment the mitochondrial fraction which was washed twice by resuspension and centrifugation at $8000 \times g$ for 10 min. The supernatant thus obtained was subsequently centrifuged at $100\,000 \times g$ for 60 min to sediment the microsomal fraction.

Enzymatic characterization of the plasma membranes

0.1 ml of the subcellular fraction suspension was added to 1 ml of the respective substrate solution, and incubated at 37 °C for 15 min except as stated. Reaction was stopped with the addition of 1 ml of ice-cold 10% trichloroacetic acid. The precipitated protein was removed by centrifugation at $1000 \times g$ for 10 min and analyzed by the method of Lowry et al. [20], using bovine serum albumin as a reference standard. Protein concentrations ranged from 100–500 μg total protein per incubation tube. The incubation mixture was then analyzed for the appropriate reaction products. A control sample was prepared by the immediate addition of 1 ml of ice cold 10% trichloroacetic acid to the subcellular fraction and substrate mixture prior to incubation. Incubation conditions were as follows (see refs 21, 16, 17):

5'-Nucleotidase (*EC* 3.1.3.5). Incubations were for 30 min or 15 min in 5 mM AMP, 100 mM glycine, 10 mM $MgCl_2$ at pH 8.5.

Glucose-6-phosphatase (*EC* 3.1.3.9). Incubation was for 15 min in 10 mM glucose 6-phosphate, 100 mM succinate–acetate buffer, pH 6.0. Inorganic phosphate was measured by the method of Fiske and SubaRow);

Succinate dehydrogenase (*EC* 1.3.99.1). 50 mM sodium succinate, 100 mM Tris buffer and 0.01% (w/v) INT (2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride, at pH 8.0 were incubated for 15 min. The reduced dye was extracted with 3 ml of ethyl acetate and the molar extinction at 490 nm determined.

Solubilization of membranes

1 mg of membranes (protein basis) were suspended in 1 ml of 50 mM sodium carbonate and 8 mg sodium dodecylsulfate and 0.1 ml β-mercaptoethanol were added. After the suspension became visually clear, it was dialized overnight against a solution of 2 M urea, 0.06% dithiothreitol (w/v) and 0.1% sodium dodecylsulfate buffered with the upper gel electrophoresis buffer (see below).

Electrophoresis

Polyacrylamide disc electrophoresis was run in 11% gels at pH 9.2. A 3.2% spacer gel, pH 6.1, was utilized, and the samples of dissolved membranes were applied in 40% sucrose. Further details are given in the description of the individual experiments.

EXPERIMENTS AND RESULTS

Preparation of membranes

Table I indicates the marker analysis for this preparation. For this table the mitochondrial and microsomal marker enzyme content of these fractions prepared as described above, succinate dehydrogenase and glucose 6-phosphatase respectively, were set at 100% and the membrane values are expressed both as specific activities

TABLE I

ANALYSIS OF LIVER CELL FRACTIONS FOR MARKER ENZYMES

Marker enzyme	Spec. act.*	Percent contamination	Enrichment
Succinate dehydrogenase	0.01 ± 0.055**	0.18	–
Glucose-6-phosphatase	9.11 ± 0.4***	7.3	–
5′-Nucleotidase	567 ± 23***	–	18

* The specific activities represent the mean ± S.D. of at least 5 different preparations.
** μmoles product produced per mg protein per h.
*** μg P_i generated per mg protein per h.

and as percentages of the control fraction. Enrichment was calculated from the specific activity of the homogenate fraction for 5′-nucleotidase. On this basis, it can be seen that the membranes have been purified about 18-fold over the homogenate, and that they are virtually free of succinate dehydrogenase. This suggests strongly that the membranes contain virtually no intact mitochondria or inner membrane fragments. In our hands, preparation of membranes as described by Neville [14], or by Berman et al. [17], consistently showed succinate dehydrogenase levels of at least 5% those found in liver mitochondria. Consistent with the enzymatic analysis, we were unable to detect any mitochondria in any membrane preparation by electron microscopy.

The primary factor in separation of the plasma membranes from mitochondria is the presence of EDTA in the density-gradient step as outlined above. Table II shows the effect of EDTA. Plasma membrane "a" was prepared by the procedure described in Methods and Materials, while plasma membrane "b" was prepared in identical fashion on the same day, without EDTA in the first layer of sucrose gradient. From the data it is clear that the presence of EDTA greatly facilitates separation of mitochondria from membranes. The basis for this observation is not presently understood, although it is possible that EDTA prevents aggregation of membrane fragments with mitochondria.

TABLE II

EFFECT OF EDTA ON MEMBRANE PREPARATION

All assays and data are expressed as described for Table I.

Membrane preparation	Marker enzyme:				
	Succinate dehydrogenase		Glucose-6-phosphatase		5′-Nucleotidase Spec. act.
	Spec. act.	%	Spec. act.	%	
Membrane "a" (+EDTA)	0.02	0.36	9.4	7.5	560
Membrane "b" (−EDTA)	1.44	2.94	17.1	13.6	254

Acrylamide electrophoresis of membrane protein

Fig. 1 is a densitometer tracing of the polypeptide pattern found for this plasma membrane preparation in sodium dodecylsulfate–polyacrylamide gels stained with Coomassie Blue. There are 30–35 separate bands, with 3 major groups of polypeptides observable. Two very large species (mol. wt $>$ 200 000) are present in major proportion, a second group of 50 000–70 000 and a third of 15 000–25 000. Within these dominant areas are many minor components, but the major groupings are generally similar to those reported by other workers for liver plasma membranes [22, 23].

Investigation of the resistance of membrane proteins to proteolytic digestion revealed a rather striking feature of the plasma membranes. Fig. 2 shows the profile obtained after digestion of the membrane with papain. It is clear that a band of molecular weight approx. 160 000 is resistant to this enzyme for at least 15 min, during which time most other polypeptides are completely digested. The group of polypeptides migrating immediately behind this unique species may also be partially resistant to the enzyme. Fig. 2 shows that the resistant polypeptide is ultimately susceptible to papain digestion but only at a much slower rate than the other proteins. However, papain treatment of detergent solubilized membrane proteins under the same concentrations and experimental conditions led to complete hydrolysis of all proteins, as did treatment of sonicated plasma membranes (Fig. 2).

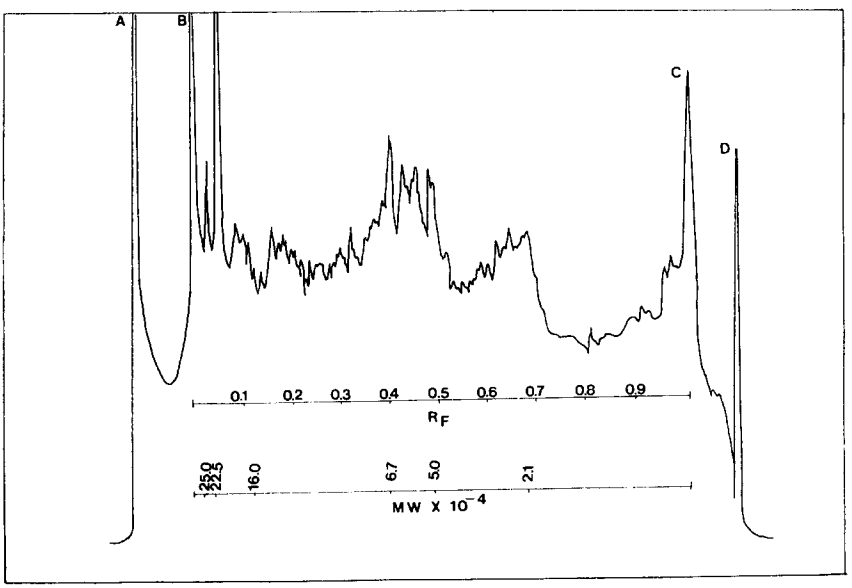

Fig. 1. Densitometer tracing of a sodium dodecylsulfate–polyacrylamide gel showing the resolution of plasma membrane proteins. 50-μg samples of membrane protein dissolved in 0.1 % sodium dodecylsulfate, 0.5 % dithiothreitol and 2 M urea, were applied to an 11 % polyacrylamide gels at pH 9.2, and electrophoresed at 1.5 mA/tube until the marker dye (bromophenol blue) reached the lower end of the gel. The gels were stained with Coomassie brilliant blue overnight, and then destained by dialysis against 7 % acetic acid. 'A' marks the beginning of the spacer gel (3.2 %) and 'B' the beginning of the lower gel. 'C' marks the tracer dye and 'D' the end of the gel. Molecular weights were determined by the migration of standards (Human γ globulin, bovine serum albumin and cytochrome *C*) in this system.

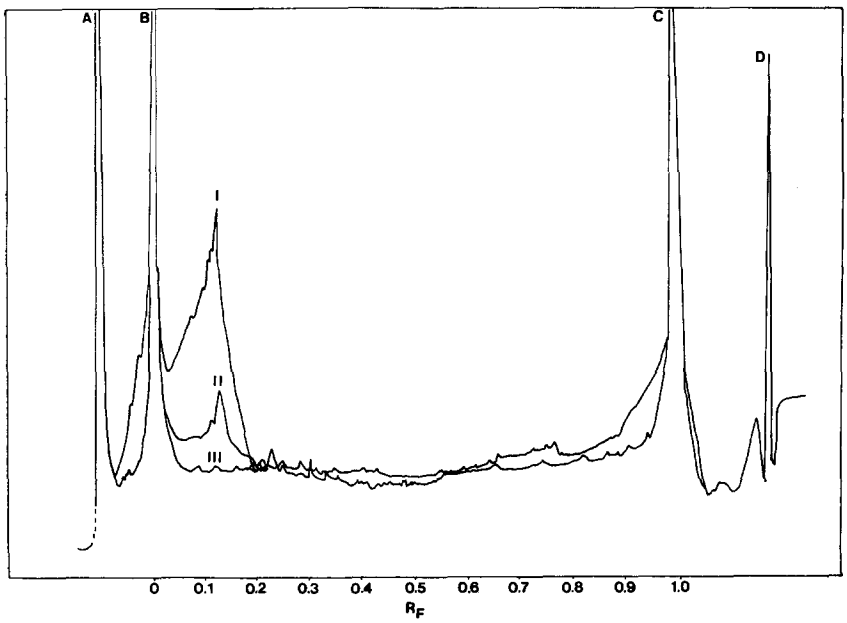

Fig. 2. Densitometer trace of polyacrylamide gels of 100 μg (original protein) of membranes exposed to papain (42 μg/ml) for: (I) 15 min; (II) 30 min and (III) membrane sonicated with a Branson Sonifier until the suspension was visually clear. Conditions of electrophoresis and labeling of the densitometer trace are the same as for Fig. 1.

Quantitative estimates of the amount of this papain-resistant protein are difficult to make since its resistance is not absolute and some digestion probably occurs even at 15 min. However, calculating from the intensity of the bands obtained from intact membranes, assuming equivalent staining for each protein, gives a value of 4–8% of the total papain-resistant material.

Deformation–electron microscopy studies

Since the data suggested that this protein is protected from papain in the intact membrane, and thus might be "buried" within the membrane matrix, further structural studies were conducted in correlation with papain digestion. Geil and his coworkers have employed a deformation technique to study the molecular organization in the structures of crystalling synthetic macromolecules [24, 25] and later extended the technique to erythrocyte ghosts [26]. This technique entails the drying of the sample on a strip of mylar film precoated with a layer of carbon and subjecting the film to a tensile stress, with the result that elongation of the mylar strip is effected. The carbon film, being quite rigid, will crack and any sample overlying the cracks will also be subjected to a tensile stress. The sample is then shadowed with evaporated platinum–carbon in a direction perpendicular to the direction of the stress, but at an elevation angle of 45°, so that any structural features revealed by the tensile stress may be shadowed and consequently observed by electron microscopy.

Although this is an indirect method, it elucidates some of the ultrastructure of the sample, thus making it possible to gain some insight into the molecular organiza-

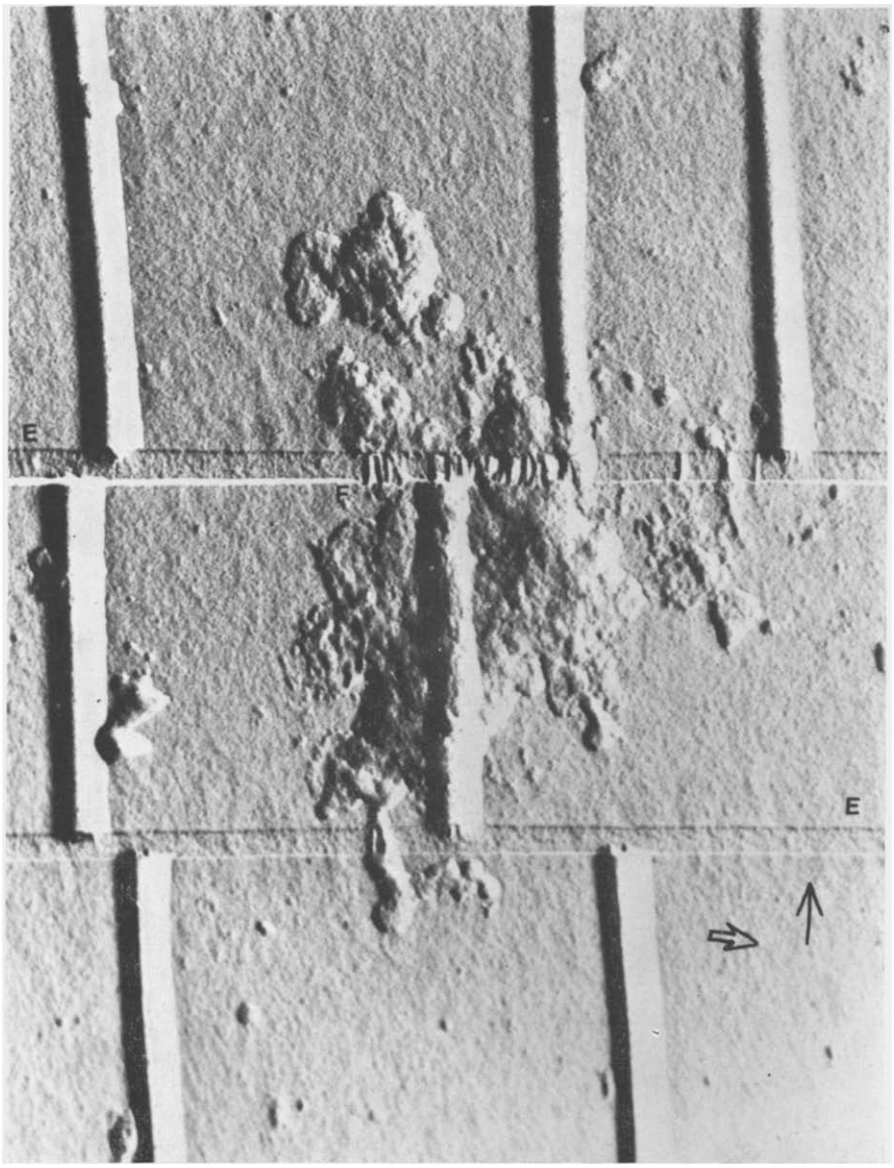

Fig. 3. Electron micrograph of liver plasma membranes after subjection to tensile stress, at a magnification of 30 000 ×. The tensile stress was applied in the direction of the solid arrow. Evaporated platinum–carbon was cast in the direction of the hollow arrow at an elevation angle of 45 °. E is a region of carbon layer where a crack has occurred. F is a region where a portion of the plasma membrane lies directly over the crack in the carbon layer, resulting in a fissure in the plasma membrane. In this figure, uniform and discrete fibrils are formed.

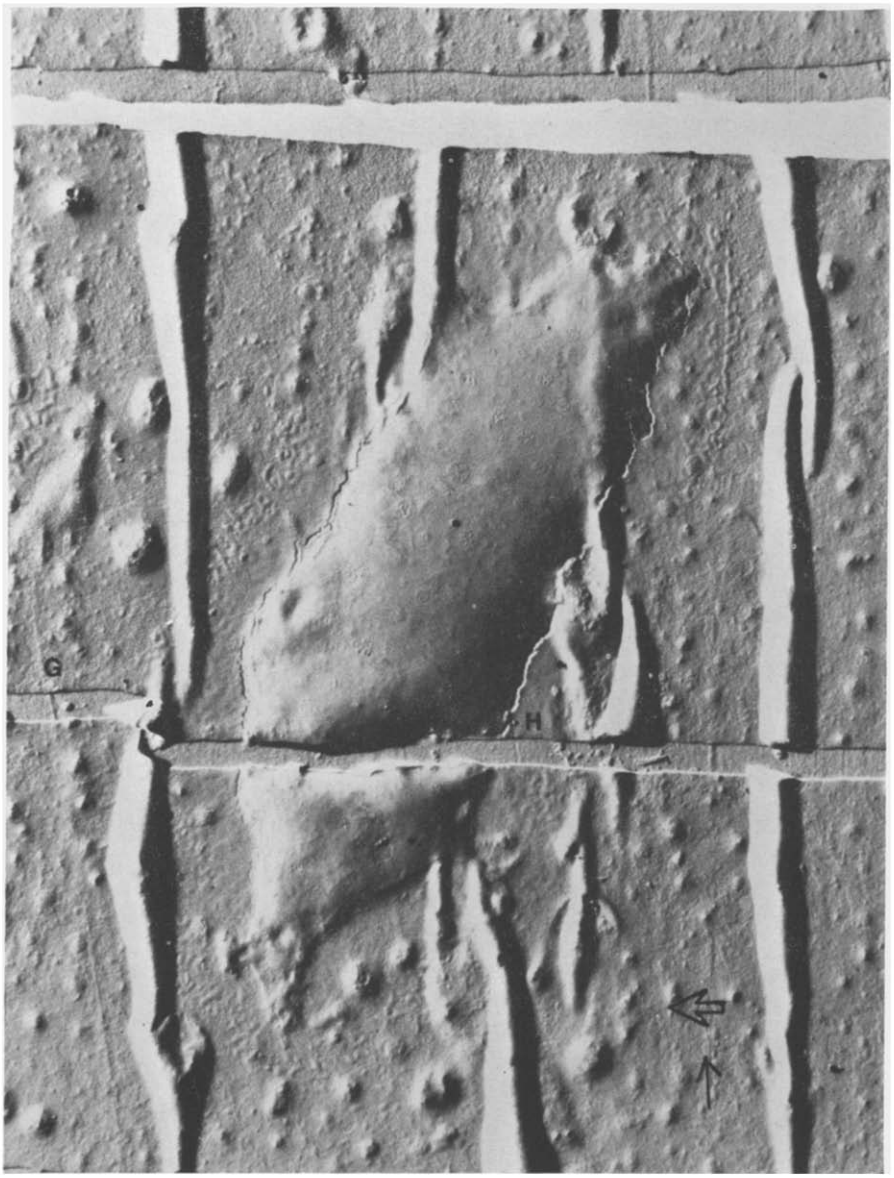

Fig. 4. Electron micrograph of papain-treated liver plasma membranes after subjection to tensile stress at a magnification of 21 000×. The tensile stress was applied in the direction of the solid arrow. Evaporated platinum–carbon was cast in the direction of the hollow arrow at an elevation angle of 45 °. G is a region of the carbon layer where a crack has occurred. H is a region where the papain-treated membranes lie directly over a crack in the carbon layer, resulting in a fissure in the membrane. There are no fibrils in the membrane fissure.

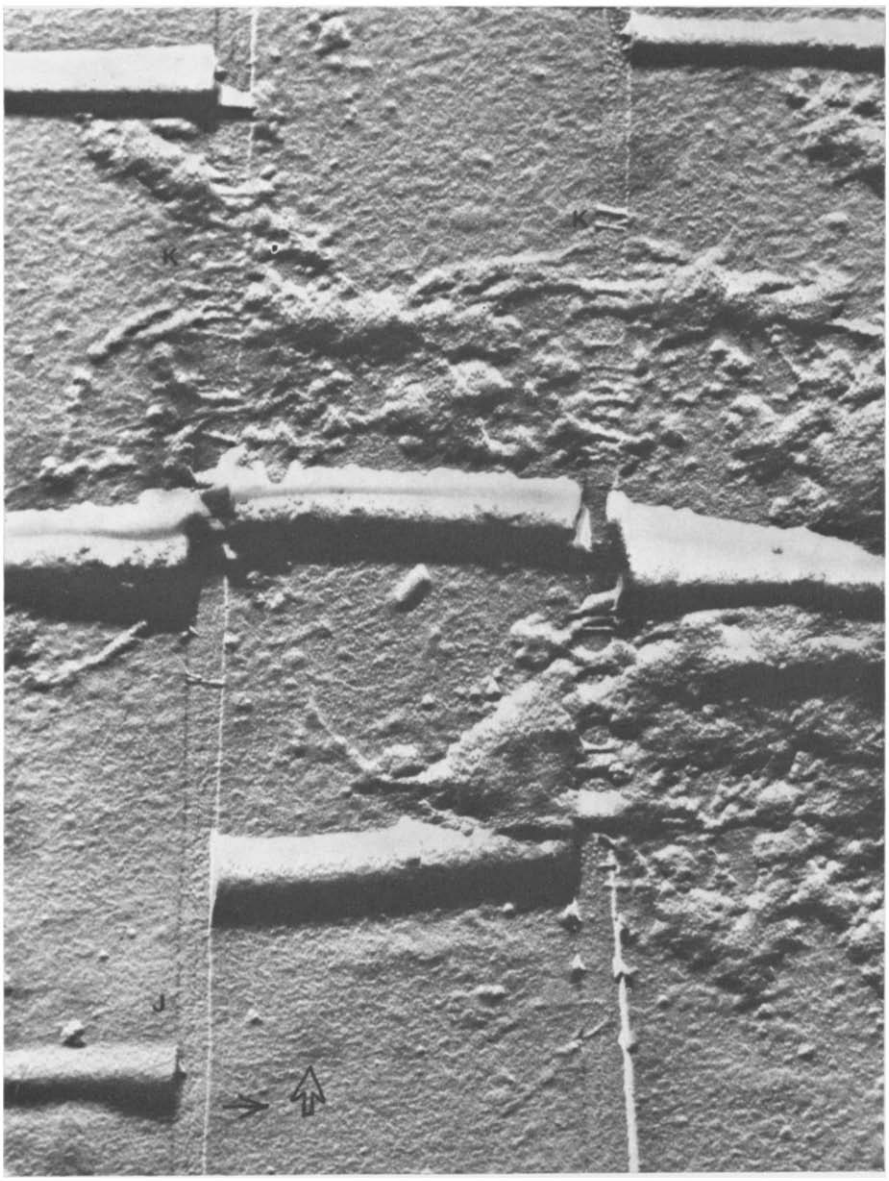

Fig. 5. Electron micrograph of liver plasma membranes treated with papain for 15 min prior to deformation, at a magnification of 30 200 ×. The tensile stress was applied in the direction of the solid arrow. Evaporated platinum–carbon was cast in the direction of the hollow arrow at an elevation angle of 45 °. 'J' is a region where a crack has occurred. 'K' is a region where the papain-treated plasma membranes lie directly over a crack in the carbon layer, resulting a in fissure in the membrane. There are fibrils formed in the membrane fissure.

tion of the ordered structures in synthetic molecules. In turn, highly interesting results are obtained when this technique is applied to biological membranes [26].

When plasma membranes were subjected to deformation, the results shown in Fig. 3 were obtained. The electron micrograph shows a region of the carbon layer clear of plasma membranes, where a crack has occurred, labeled as E. Another region where membranes lie directly over a crack in the carbon layer, is labeled as F. At the point of fissure of the membranes, uniform and discrete fibrils or rods are seen extending across the membrane fissures. These fibrils are approximately 1400 Å long and 200 Å in diameter.

To determine whether these fibrils were protein, a few drops of papain suspension were placed over a dried sample of plasma membranes and allowed to dry overnight. As shown in Fig. 4, this treatment eliminated the fibrils completely. In addition, the surface of the membrane has taken on a smooth appearance. The small (approx. 100 Å) particle on the membrane surface and in the surrounding area are probably individual papain molecules, since electron micrographs of the enzyme along revealed similar particles as well as larger aggregates.

Plasma membranes treated with papain for 15 min are shown in Fig. 5. A reduction in the number of fibrils in the membrane fissures is apparent, but there are still many fibrils present. This is the same membrane preparation which revealed almost total loss of protein except for the 160 000-mol. wt unit in Fig. 2.

DISCUSSION

Although the method for preparation of plasma membranes is not the major point of this report, it does deserve some comment. Many other methods have been reported for the preparation of plasma membranes from rat liver and, as is the case for our preparation, most of these techniques are modifications of earlier procedures worked out by the pioneers in this field, Neville [13] and Emmelot [15]. Very recently, a report on a rapid and more powerful preparation technique utilizing an aqueous, two-phase polymer system has appeared [18]. This method yields more than twice the amount of membrane per gram of liver when compared to most of the earlier reports. However, in all the methods described to date [13–19, 28], at least 3–5% of the fraction of a protein basis consists of mitochondria as determined by analysis of mitochondrial marker enzyme. Therefore, it appears that the major significance of the present work in this regard is the development of a technique for virtually eliminating mitochondria, at least as far as detection of marker enzymes is concerned. In addition, the method is relatively simple, reproducible and yields 1–2 mg membrane protein per g of fresh tissue.

The membrane profile from the sodium dodecylsulfate–polyacrylamide gel electrophoresis resembles that reported by other workers for liver plasma membranes. We find two very intense bands in the high-molecular weight range, (approx. 250 000 and 225 000 as reported by Neville for rat liver [22]). Whether these bands resemble the Tektin A found in the erythrocyte [2, 27] is not known, but the molecular weights are similar. One or two dominant high-molecular weight proteins have been observed in many plasma membrane preparations from a variety of sources.

The group of four proteins of molecular weight ranging from 67 000–50 000 is also commonly observed in plasma membrane fractions. Neville [22] reported a

common protein of molecular weight of 48 000 in erythrocyte, kidney and liver plasma membranes. In fact, it appears that this group of proteins is perhaps the most readily distinguishable characteristic for the liver plasma membrane. In this regard, we might point out that in our data (Fig. 1), the 67 000-mol. wt protein is the most intense in this group while in the data of Neville [22] and of Dehlinger and Schimke [23], the 50 000-mol. wt component appears most prominent. Since mitochondria also show a prominent 50 000-mol. wt protein [22, 23], it seems possible that the presence of contaminating mitochondria in the earlier membrane preparations account for the increased intensity of this band when compared to our membrane preparation which is free of mitochondrial contamination. Finally, we also find a group of small molecular weight proteins with a dominant peak at 21 000 as reported by others.

Of greatest interest is the demonstration of a relatively papain-resistant protein in the plasma membranes. Since this protein is digested readily by papain when the membranes are disrupted by detergent or sonication, the data suggest that this protein or group of proteins are protected from the enzyme by virtue of their structural relationship in the membrane. In particular, it seems likely that this papain-resistant protein(s) is protected within the matrix of the membrane. Since the membrane sheets and vesicles are prepared by homogenization, it seems unlikely, for example, that one side of a membrane would be dominantly shielded; i.e. that the papain would have access to only one side of the membranes. This belief would appear to gain support by the observation that all the membrane protein is hydrolyzed by 45 min of papain treatment.

It is also clear that we are observing the degradation of a species whose original molecular weight was approx. 160 000 rather than generation of a fragment of this size from some larger protein. In the latter case it would be expected that we would observe a time-dependent decrease in molecular weight of the resistant protein rather than a decrease in the intensity at a fixed molecular weight as is shown in Fig. 2.

One potential implication of these results, of course, is that these papain-resistant proteins may serve a structural function since they may be buried within the membrane. Further evidence to support this view comes from the electron micrographs of structurally deformed membranes. The existence of membrane fibers revealed by this technique has been reported for other biological membrane preparations such as the erythrocytes and chloroplasts [Geil, P. H., unpublished]. It is not presently known whether pre-existing fibers are revealed by the deformation or whether they are generated by stretching some originally non-fibrous molecules. In any case, it has been demonstrated that the fibers are probably protein in nature since they are found in lipid-depleted membranes [ref. 26 and Geil, P. H., unpublished) and as shown in Fig. 5 are eliminated by digestion of the membranes with papain. However, we do not imply that these fibers are individual protein molecules since the dimensions appear inappropriate for such an interpretation (1400 Å $\times$ 200 Å). Thus, although the data presented here in no way establish that the papain-resistant protein found in the liver plasma membrane is directly responsible for the fibrils found in mechanically deformed membranes, it is of interest that in membranes treated for 15 min with papain such fibrils are still prominent even though the vast majority of the membrane protein has been digested.

ACKNOWLEDGEMENTS

This investigation was supported by Grant No. 14-01-0001-1285 from the Office of Saline Water.

J.E.Z. is a recipient of Career Development Award, No. AM70031.

REFERENCES

1 Guidotti, G. (1972) Annu. Rev. Biochem. 41, 731–752
2 Bretscher, M. S. (1973) Science 181, 622–629
3 daSilva, D. D. and Branton, D. (1970) J. Cell Biol. 45, 598–605
4 Shapiro, A. L., Viñuela, E., Maizel, J. V. (1967) Biochem. Biophys. Res. Commun. 28, 815–820
5 Berg, H. C. (1969) Biochim. Biophys. Acta 183, 65–78
6 Neville, D. M., Jr (1971) J. Biol. Chem. 246, 6328–6334
7 Maddy, A. H. (1964) Biochim. Biophys. Acta 88, 390–399
8 Berg, H. C. (1969) Biochim. Biophys. Acta 183, 65–78
9 Bretscher, M. S. (1971) J. Mol. Biol. 58, 775–781
10 Rifkin, D. B., Compans, R. W., Reich, E. (1972) J. Biol. Chem. 247, 6432–6437
11 Phillips, D. R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284–289
12 Bender, W. W., Garan, H. and Berg, H. C. (1971) J. Mol. Biol. 58, 783–797
13 Neville, D. M., Jr (1960) J. Biophys. Biochem. Cytol. 8, 413–422
14 Neville, D. M., Jr (1968) Biochim. Biophys. Acta 154, 540–552
15 Emmelot, P., Bos, C. J., Benedetti, E. and Rumke, D. (1964) Biochim. Biophys. Acta 90, 126–145
16 Ray, T. K. (1970) Biochim. Biophys. Acta 196, 1–9
17 Berman, H. M., Gram, W. and Spirites, M. A. (1969) Biochim. Biophys. Acta 183, 10–18
18 Lesko, L., Donlon, M., Marinetti, G. V. and Hare, J. D. (1973). Biochim. Biophys. Acta 311, 173–179
19 Dorling, D. R. and LePage, R. H. (1973) Biochim. Biophys. Acta 318, 33–44
20 Lowry, D. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21 Weaver, R. A. and Boyle, W. (1969) Biochim. Biophys. Acta 173, 377–388
22 Neville, D. M., Jr (1971) J. Biol. Chem. 246, 6335–6338
23 Dehlinger, P. J. and Schimke, R. T. (1971) J. Biol. Chem. 246, 2574–2583
24 Geil, P. H. (1963) Polymer Single Crystals, pp. 445–452, Interscience, N.Y.
25 Garber. C. A. and Geil, D. H. (1968) Mackromol. Chem. 113, 251–269
26 Blais, J. J. B. P. and Geil, D. H. (1969) Biopolymers 8, 275–287
27 Clarke, M. (1971) Biochem. Biophys. Res. Commun. 45, 1063–1070
28 Nigam, V. H., Morais, R. and Karasaki, S. (1971) Biochim. Biophys. Acta 247, 34–40