Plasma Membranes from Experimental Granulation Tissue

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1. A procedure was developed for the preparation of plasma membranes from experimental granulation tissue of the rat without the addition of enzymes. The yield is better than 20% and the purification at least tenfold. 2. Values are given for the activities of 5'-nucleotidase, Na^+ , K^+ -activated Mg^{2+} -dependent adenosine triphosphatase and leucine β -naphthylamidase, for lipid composition, and for the gel-electrophoretic patterns of proteins and glycoproteins in the membrane preparations. 3. The plasma membranes from the mature granulation tissue contain proportionally more protein in the lipid phase, but the specific activities of 5'-nucleotidase and Na^+ , K^+ -activated Mg^{2+} -dependent adenosine triphosphatase are smaller than in the proliferating tissue. Certain differences were repeatedly observed in the gel-electrophoretic patterns of the developmental phases. 4. The plasma membranes from the granulation tissue were compared with those from rat peritoneal macrophages and from embryonic-chick tendon cells.

Much work has been done on the isolation and characterization of plasma membranes from cultured fibroblasts (Warren *et al.*, 1967; Perdue & Sneider, 1970; Gahmberg & Simons, 1970; Graham, 1972; Bingham & Burke, 1972; Scher & Barland, 1972; Lelievre, 1973; Smith & Crittenden, 1973; Israel *et al.*, 1973; Wray & Perdue, 1974). The purpose of the present work was to study the plasma membranes of fibroblasts obtained from solid connective tissue and to compare them in the proliferative and mature, collagen-synthesizing phases (Lampiaho & Kulonen, 1967). There is an extensive literature on the relation between the cell growth and the structure of plasma membranes, especially with reference to contact inhibition, virus transformation and cancer (cf. Hakomori *et al.*, 1972; Warren *et al.*, 1973). For studies on adhesion, fusion and feedback inhibition of the fibroblasts, the intact plasma membranes from fixed tissue may be more relevant than those from cultured cells.

Experimental

Production of materials

Granulation tissue. Female albino rats of the Wistar strain (2–3 months old, weight 130–200g) were kept on a standard laboratory diet (from Messrs. Hankkija, Helsinki, Finland) supplemented with vegetables. Solyom & Lauter (1973) have reported that the activities of plasma-membrane enzymes are higher in males than in females. Growth of the granulation tissue was induced by pieces of viscose cellulose sponge (dry size 4 mm $\times$ 14 mm $\times$ 14 mm; dry weight 60–70 mg), made by Kongsfoss Fabrikker

(Bygdøy allé 5, Oslo, Norway), which had been sterilized by boiling in 0.9% NaCl for 30 min. Six pieces were implanted symmetrically under the dorsal skin of each rat (Viljanto & Kulonen, 1962; Viljanto, 1964). The granulomas were removed in the proliferative phase, after an implantation period of 7 days, or in the mature phase, after 21 days (Lampiaho & Kulonen, 1967; Kulonen, 1970). The surrounding capsules were dissected off and the granulomas immersed immediately into ice-cold iso-osmotic (8.54%, w/v) sucrose. The best results are achieved with fresh granulomas, but frozen tissue can also be used.

Tendon cells. The embryonic-chick tendon cells were prepared from about 250 17-day-old chick embryos by using trypsin, trypsin inhibitor and collagenase as described in detail by Dehm & Prockop (1971).

Peritoneal macrophages. The macrophages were obtained from adult albino rats, which had received 3 days previously an intraperitoneal injection of 5 ml of semi-fluid thioglycollate medium (Messrs. Orion, Helsinki, Finland). The macrophages were isolated in a Ficoll (Pharmacia, Uppsala, Sweden) gradient by the method of Zembala & Asherson (1970).

Methods used in the preparation of plasma membranes

Experimental granulation tissue. For the plasma membranes from fixed tissue to remain comparable with those from cultured cells the surface coating must be left intact. Both enzymic liberation of the cells and pretreatment with heavy metals have therefore to be avoided. The whole procedure was carried out at 4°C unless stated otherwise.

The tissue was homogenized into iso-osmotic (8.54%, w/v) sucrose as described by Allan & Crumpton (1970), centrifuged at 7000g for 30 min and the resuspended sediment applied over a continuous sucrose gradient which was formed by pipetting 2 ml each of 40, 38, 36, 34, 32 and 28% (w/v) sucrose solutions into cellulose acetate tubes of a swing-out rotor (MSE 50, 3×23 ml) and left for 1–2 h. During the centrifugation at 100000g, for 4 h, 86% of the total 5'-nucleotidase activity moved to the density of 28–38% sucrose, but 73% of succinate dehydrogenase was found in the 38% sucrose or in the bottom of the tube (Fig. 1).

We also used the Ficoll gradient as described by Gahmberg & Simons (1970) after a homogenization as described by Allan & Crumpton (1970). The purification was good, but the yield only about 5%. The final preparative procedure, a compromise between the yield and the purification, is described in the Results section.

Embryonic tendon cells and peritoneal macrophages. The isolated cells were disintegrated by freezing and thawing four times in water containing 2 mM-EDTA and homogenized with the tight-fitting Dounce homogenizer by using 40–100 strokes. The rupture of cells was confirmed by microscopic examination. The isolation of plasma membranes was carried out in a discontinuous sucrose gradient [3 ml each of 20, 28, 38, 50 and 70% (w/v) sucrose, in water, at 25000g for 2 h]. The fraction at the interphase of 28 and 38% sucrose contained plasma membranes, as ascertained

by measuring the activities of 5'-nucleotidase, Na⁺,K⁺-activated Mg²⁺-dependent adenosine triphosphatase and leucine β -naphthylamidase.

Analytical methods

Enzyme assays. The specific activities were expressed as the amount of reaction product liberated in μ mol/h per mg of protein at 37°C $\pm$ s.d. (*n*). The methods used are listed as follows. (a) 5'-Nucleotidase (EC 3.1.3.5); Michell & Hawthorne (1965); $\pm 10.8\%$ (10). (b) Acid phosphatase (EC 3.1.3.2); Gianetto & de Duve (1955); $\pm 9.6\%$ (10); β -glycerophosphate was used as the substrate and alkaline phosphatase was inhibited with 4 mM-EDTA (Hübscher & West, 1965). (c) Na⁺,K⁺-activated Mg²⁺-dependent adenosine triphosphatase (EC 3.6.1.3); Swanson *et al.* (1964); $\pm 6.5\%$ (10). (d) Glucose 6-phosphatase (EC 3.1.3.9); Hinton *et al.* (1970); $\pm 7.0\%$ (10); the acid and alkaline phosphatases were inhibited with 2 mM-KF and 4 mM-EDTA respectively (Hübscher & West, 1965). (e) Leucine β -naphthylamidase (EC 3.4.11.1); Goldberg & Rutenburg (1958); $\pm 8.5\%$ (10). (f) Succinate dehydrogenase (EC 1.3.99.1); Earl & Korner (1965); $\pm 15.0\%$ (10).

Chemical methods. P_i liberated in the enzyme assays was determined as described by Martin & Doty (1949) [s.d. $\pm 4.6\%$ (10)]. The protein contents were determined by the method of Lowry *et al.* (1951) as modi-

Fig. 1. Centrifugation of granuloma homogenate in continuous buffered sucrose gradient

Frozen 21-day granulomas were homogenized in iso-osmotic sucrose solution and the homogenate was centrifuged at 7000g for 30 min. The resuspended sediment was fractionated in a swing-out rotor in a linear unbuffered sucrose gradient. For the details see the text. ●, 5'-Nucleotidase; ○, succinate dehydrogenase; both as μ mol/h per mg of protein; ■, sucrose (% w/v).

fied by Pikkarainen (1968), with bovine albumin as the standard [s.d. $\pm 2.0\%$ (10)].

For the determination of phospholipids and cholesterol, the plasma membranes were sedimented and washed twice with water by centrifuging at 100000g for 40 min. The pellet was homogenized in 0.5 ml of water and the protein determined in a portion. The rest of the pellet was freeze-dried and extracted three times for 24 h with chloroform-methanol (2:1, w/v) at room temperature by the method of Folch *et al.* (1957). The chloroform-methanol extracts were examined on silica-gel G plates (E. Merck AG, Darmstadt, Germany) by t.l.c. For cholesterol, the silica-gel G plates were developed with light petroleum (b.p. 60–80°C)-diethyl ether-acetic acid (85:15:1, by vol.) and for phospholipids with chloroform-methanol-formic acid-water (291:291:6:13, by vol.). Cholesterol was determined as described by Fieser (1953), s.d. $\pm 2.6\%$ (10), and phospholipids were measured by the method of Bartlett (1959) after the combustion of phosphorus by the method of Svanborg & Svennerholm (1961), s.d. $\pm 1.6\%$ (10).

Sucrose concentration. The concentrations and densities of the sucrose and Ficoll (Pharmacia, Uppsala, Sweden) solutions were determined by refractive index with an ATAGO no. 15050 refractometer and calculated by the method of Dobrota (1971), s.d. $\pm 0.4\%$ (10).

Gel electrophoresis. Plasma-membrane preparations were sedimented at 100000g for 40 min. The pellets were homogenized in 1–2 ml of 8M-urea–2% (w/v) sodium dodecyl sulphate–2% (w/v) mercaptoethanol with small homogenizers (vol. 0.5 ml; radial clearance about 0.075 mm). About 50 μ g of protein was used for protein and about 250 μ g for glycoprotein runs. The samples were heated at 100°C for 3 min to inactivate the enzymes.

The 5% (w/v) polyacrylamide gels with 4.06% cross-linking were prepared by mixing the stock solutions A, B and C and water in the ratio 6:4.7:2:3, by vol. (W. H. Evans, personal communication). Solution A contained 2.34 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 4.07 g of anhydrous Na_2HPO_4 , 0.5 g of sodium dodecyl sulphate (BDH Chemicals Ltd., Poole, Dorset, U.K.; no. 30176, extra pure), 15 g of urea and 0.25 ml of *NNN'*-tetramethylethylenediamine in 125 ml of water. Solution B contained 2.03 g of *NN'*-methylenebisacrylamide (Eastman Kodak Co., Rochester, N.Y., U.S.A.; no. 8383) and 50 g of acrylamide (Fluka AG, Buchs, Switzerland; no. 01700) in 300 ml of water. Solution C contained 0.38 g of ammonium persulphate in 50 ml of water. Acrylamide and *NN'*-methylenebisacrylamide were purified as described by Loening (1967). The cylindrical gels (0.4 cm $\times$ 6.5 cm) were formed by the polymerization of acrylamide in glass tubes for at least 30 min.

The buffer contained 5 g of sodium dodecyl sul-

phate, 23.4 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 125 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 5 litres of water. Electrophoresis was carried out at room temperature for 2.5 h at a constant current of 8 mA/gel, 30 V. The following proteins were used as references in assessing the molecular weights: thyroglobulin (from Dr. K. Simons, Helsinki, Finland; mol.wt. 160000); neuraminidase (Behring-Werke A.G., Marburg/Lahn, Germany; mol.wt. 90000); trypsin (Novo, Copenhagen, Denmark; mol.wt. 23500); cytochrome c (Fluka A.G., mol.wt. 12000).

Proteins were stained by the method of Lenard (1970) and glycoproteins by that of Glossmann & Neville (1971). The stained gels were scanned on a Perkin-Elmer UV-VIS 139 spectrophotometer by using an adaptor made in our workshop and a Servogor RE 514.9 recorder.

Electron microscopy

Membrane pellets were fixed overnight in 3% glutaraldehyde in 0.2M-sodium cacodylate buffer, pH 7.25, post-fixed for 30 min in 1% OsO_4 in the same buffer and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead hydroxide and studied in a Siemens Elmiskop 1 A electron microscope (Laboratory of Electron Microscopy, University of Turku).

Results

Preparation and properties of plasma membranes from granulation tissue

Standard procedure. Fig. 2 shows the flow-sheet. Usually a sample of about 20 g of fresh granulation tissue was processed. It was immersed in iso-osmotic (8.54%, w/v) sucrose and forced in lots of 3 g through a perforated plate of 3 cm diameter by the press designed by Allan & Crumpton (1970) and made in our workshop. The holes had a diameter of 0.75 mm and were spaced at a distance of 1.50 mm from centre to centre. The milky suspension that emerged through the plate was filtered through four layers of nylon cloth and the filtrate homogenized with five strokes in either a loose- or tight-fitting Dounce homogenizer (radial clearances 0.225 and 0.075 mm respectively).

If necessary, frozen granulomas could also be used, but the yield in terms of the activities of 5'-nucleotidase and K^+ , Na^+ -activated Mg^{2+} -dependent adenosine triphosphatase was decreased by about one-half. The purification factor was less affected by the freezing than was the yield. The plasma membrane from frozen tissue presumably fragmented to smaller pieces, which were partially lost during the preparation.

The yield was better with the tight-fitting Dounce homogenizer than with the loose-fitting one, except

Fig. 2. Flow-sheet for the standard procedure for the preparation of plasma membranes from granulation tissue

For the details see the text.

when calculated in terms of leucine β -naphthylamidase. The clearance of the homogenizer did not affect the degree of purification, except with leucine β -naphthylamidase, for which the larger clearance was favourable.

For the initial removal of the microsomal fraction, the whole homogenate was centrifuged at 7000g for 30 min. The pellet was resuspended in iso-osmotic (8.54%, w/v) sucrose with 15 strokes in a Dounce homogenizer.

After centrifugation of the homogenate in a continuous sucrose gradient, the highest specific activities of 5'-nucleotidase were found in the sucrose concentrations of about 30–40% (cf. Fig. 1). To improve the exclusion of mitochondria the 38% (w/v) sucrose was chosen to be the limit used in the preparative work. The centrifugation was carried out in an MSE 65 ultracentrifuge (3 $\times$ 23 ml swing-out rotor) at 100 000g for 4 h. The subfractions were collected from the interphases by suction. For large amounts of tissue a zonal rotor can be used.

The presence of 10 mM-Tris-HCl buffer, pH 7.4, did not make any significant difference, but the addition of 5 mM-Mg²⁺ rendered the separation of the plasma membranes from mitochondria impossible.

Purity and yield of the preparations. Table 1 shows that the interphases of 20/28% and 28/38% sucrose

Table 1. Specific activities of the marker enzymes and the distribution of protein in the subcellular fractions

Fresh 21-day granulomas were homogenized and fractionated as shown in Fig. 2. The clearance of the Dounce homogenizer was 0.075 mm. The means $\pm$ s.e.m. ($n = 4-7$) of the specific activities (μ mol of reaction product/h per mg of protein at 37°C) are given; the distribution of protein is expressed as a percentage of that in the original homogenate. Abbreviation: ATPase, adenosine triphosphatase.

Fraction	5'-Nucleotidase	Na ⁺ , K ⁺ -activated Mg ²⁺ -dependent ATPase	Leucine β -naphthylamidase	Acid phosphatase	Glucose 6-phosphatase	Succinate dehydrogenase	Protein
Whole homogenate	0.34 $\pm$ 0.03	2.44 $\pm$ 0.11	0.49 $\pm$ 0.04	0.94 $\pm$ 0.06	0.26 $\pm$ 0.03	0.07 $\pm$ 0.02	100
7000g supernatant	0.44 $\pm$ 0.06	1.62 $\pm$ 0.07	0.42 $\pm$ 0.03	0.79 $\pm$ 0.03	0.24 $\pm$ 0.03	0.00	68
7000g sediment	1.04 $\pm$ 0.08	4.54 $\pm$ 0.22	0.80 $\pm$ 0.08	1.64 $\pm$ 0.11	0.31 $\pm$ 0.02	0.24 $\pm$ 0.04	21
<20% sucrose	0.76 $\pm$ 0.15	1.42 $\pm$ 0.27	0.49 $\pm$ 0.19	1.11 $\pm$ 0.20	0.46 $\pm$ 0.11	0.00	5
20/28% sucrose*	1.42 $\pm$ 0.13	9.00 $\pm$ 0.81	2.12 $\pm$ 0.31	1.36 $\pm$ 0.11	0.24 $\pm$ 0.07	0.01 $\pm$ 0.01	2
28/38% sucrose†	3.40 $\pm$ 0.23	23.98 $\pm$ 1.60	5.31 $\pm$ 0.52	3.41 $\pm$ 0.29	0.39 $\pm$ 0.04	0.12 $\pm$ 0.02	2
>38% sucrose	0.93 $\pm$ 0.12	3.50 $\pm$ 0.22	0.74 $\pm$ 0.09	1.63 $\pm$ 0.08	0.23 $\pm$ 0.04	0.59 $\pm$ 0.07	9

* 'Light' plasma membranes; † 'Heavy' plasma membranes.

Table 2. *Yield and purification of plasma membranes*

The yields are expressed as the percentage that the total activity of the indicated enzyme is of the respective activity in the whole homogenate. The purification is calculated from the corresponding specific activity per unit of protein. The means $\pm$ S.E.M. ($n = 3-6$) are given.

Basis of calculation		Yield (%)			Purification (fold)			
		7-day granuloma		21-day granuloma	7-day granuloma		21-day granuloma	
		Fraction ...	'Heavy'	'Light'	'Heavy'	'Heavy'	'Light'	'Heavy'
5'-Nucleotidase			5 ± 1	4 ± 1	19 ± 1	14 ± 1	4 ± 1	11 ± 2
Na ⁺ ,K ⁺ -activated Mg ²⁺ -dependent adenosine triphosphatase			11 ± 0	4 ± 0	18 ± 2	29 ± 8	4 ± 0	10 ± 1
Leucine β-naphthylamidase			6 ± 1	5 ± 1	18 ± 2	15 ± 2	5 ± 2	10 ± 1
Acid phosphatase				1 ± 0	6 ± 1			
Glucose 6-phosphatase				1 ± 0	2 ± 0			
Succinate dehydrogenase				0.4	0.1			
Protein				2 ± 0	2 ± 0			

Table 3. *Lipids in plasma membranes from granulomas at various phases*

The plasma membranes were prepared as shown in Fig. 2, sedimented, washed twice with water and freeze-dried. Total phospholipid + cholesterol is taken as the reference (=1.00) for the calculation of the molar fractions.

Lipid	Fraction ...	Molar fractions			Lipid/protein (μ mol/mg)	
		7-day granuloma	21-day granuloma	21-day granuloma	7-day granuloma	21-day granuloma
		'Heavy'	'Light'	'Heavy'	'Heavy'	'Heavy'
Cholesterol (free)		0.40	0.35	0.43	0.78	0.53
Total phospholipid		0.60	0.65	0.57	1.19	0.68
Sphingomyelin		0.11	0.07	0.14	—	—
Phosphatidylcholine		0.21	0.34	0.23	—	—
Phosphatidylethanolamine		0.13	0.13	0.12	—	—
Lysophosphatidylcholine		0.03	0.03	0.02	—	—
Phospholipid + cholesterol		1.00	1.00	1.00	1.98	1.20

contained enriched activities of the marker enzymes of plasma membranes. The ratios between the activities in the 20/28% to those in 28/38% fractions were constant. There was some succinate dehydrogenase in the 28/38% sucrose fraction but less than in the 7000g sediment. In the same fraction there was little abundance of glucose 6-phosphatase to indicate the presence of the endoplasmic reticulum; although some acid phosphatase was present it does not necessarily prove contamination with lysosomes (Gahmberg & Simons, 1970). Judged from the protein content the fractions sedimenting through the 38% sucrose also contain an appreciable amount of plasma membranes but mixed with other subcellular matter.

The purification, calculated on the basis of activities of the marker enzymes, was about tenfold in the 'heavy' (28/38%, w/v, sucrose interface) plasma membranes from the mature 21-day granulomas (Table 2). The purification was much lower in the 'light', or 20/28% sucrose-interface fraction. The

total yield was more than 20%, which is rather satisfactory. In proliferating granuloma the yield was lower but the purification better than in the respective samples from mature granulomas (see the Discussion section). The possibility of a further purification was checked by the re-centrifugation of the 28/38% sucrose fraction in a linear sucrose gradient of 28–40% (w/v) sucrose. No improvement in the specific activities was observed. The highest specific activities of the enzymes were observed at a density of 1.106, but the yields of these enzymes were largest at density 1.120, which corresponds to the values observed by Lelievre (1973), Smith & Crittenden (1973) and Wray & Perdue (1974).

The adenylate cyclase activities of these preparations were assessed by M. Viheraari (unpublished work). The purification in the 20/28% sucrose fraction was about sixfold and in the 28/38% sucrose fraction about 19-fold. In all the fractions from 3-week granulomas the specific activities were consistently higher than in those from 1-week granulomas.

Fig. 3. Gel-electrophoretic distribution of proteins from plasma membranes of proliferating and mature granulomas

A sample containing 50 μ g of protein was dissolved in urea-mercaptoethanol-sodium dodecyl sulphate solution and fractionated by electrophoresis in polyacrylamide gels. For the details see the text. The colour density patterns (E_{570} , light-path 0.4 cm) after protein staining are shown. The same patterns are presented in Fig. 5 (GR-7, GR-21) with the molecular weights indicated. The peaks are numbered as in Figs. 4 and 5. The molecular weights of the peaks 4, 5, 7, 12 and 15 were estimated as 100 000, 76 000, 56 000, 25 000 and 12 000 respectively, by comparison with standards.

Fig. 4. Gel-electrophoretic distribution of glycoproteins from plasma membranes of proliferating and mature granulomas

The electrophoresis of samples (250 μ g of protein) was carried out as described in the legend to Fig. 3 and in the text. Staining was by the method of Glossmann & Neville (1971). The peaks are numbered as in Figs. 3 and 5.

Composition. Table 3 shows the molar ratios of various lipids in the plasma-membrane fractions. The typical abundance of cholesterol (almost all free), phosphatidylcholine and sphingomyelin (Renkonen *et al.*, 1970) was observed and it even increased from 7-day to 21-day granulomas. The lipids in the 'light' fraction were less characteristic for membranes than those in the 'heavy' one (see the Discussion section).

The gel-electrophoretic patterns of proteins (Fig. 3) and glycoproteins (Fig. 4) were constant. About 20 protein bands were observed, with molecular weights 12 000–160 000, which latter band may contain larger molecules also (Greenberg & Glick, 1972). Four or five glycoproteins could be separated ranging from 40 000 to 117 000 in molecular weight. Peak no. 17 may be a glycolipid. The present results and those of Wray & Perdue (1974) on cultured chick-embryo fibroblasts are in essential agreement.

Electron microscopy. Both the 'light' and the 'heavy' plasma membrane fractions were found to contain mainly thin-walled vesicles and sheets (Plate 1). No nuclei, mitochondria or rough endo-

plasmic reticulum were present in these fractions. The vesicles in the 'light' fraction were generally smaller than those in the 'heavy' fraction. Some vesicles in both fractions contained amorphous electron-dense material.

Plasma membranes from embryonic-chick tendon cells and rat peritoneal macrophages

The specific activities of the marker enzymes of plasma membranes isolated from embryonic-chick tendon cells, which produce almost entirely collagen, are shown in Table 4. They are very low, but it is not known whether the enzymic treatments during the isolation of the tendon cells have affected the surface enzymes. The mutual ranking of the enzyme activities in the plasma membranes of the rat peritoneal macrophages does not resemble that in the granuloma or tendon cell preparations. Another difference between granuloma and tendon is demonstrated in the gel-electrophoretic protein patterns as shown in Fig. 5. At least superficially there is more similarity between the plasma membranes from tendon cells and macrophages.

EXPLANATION OF PLATE I

Electron-microscopic appearance of the 'heavy' plasma membrane fraction from mature granulomas

The preparation of the fraction and the staining are described in the text. Magnification $\times 27000$. The bar represents $1\ \mu\text{m}$.

Table 4. Specific activities of the marker enzymes from plasma membranes isolated from experimental granulation tissue, embryonic-chick tendon cells and rat peritoneal macrophages

The preparations and assay methods are described in the text. Results (μ mol of the reaction product/h per mg of protein at 37°C), mean values of duplicate assays, and from granulomas ('heavy' fraction) also the s.e.m. ($n = 3-7$) are given.

Origin of plasma membranes	5'-Nucleotidase	Na ⁺ ,K ⁺ -activated Mg ²⁺ -dependent adenosine triphosphatase	Leucine β -naphthylamidase
Experimental granuloma:			
7-day	10.20 $\pm$ 0.20	47.00 $\pm$ 3.39	3.48 $\pm$ 0.49
14-day	5.2	56.2	6.0
21-day	3.40 $\pm$ 0.23	24.00 $\pm$ 1.60	5.31 $\pm$ 0.52
Embryonic-chick tendon cells	0.9	5.4	2.0
Rat peritoneal macrophages	13.7	12.3	6.0

Fig. 5. Gel-electrophoretic distribution of proteins from experimental granulation tissue, embryonic-chick tendon cells and rat peritoneal macrophages

The experimental details are described in the text (cf. Figs. 3 and 4). The molecular weights (in thousands) are indicated, as are also the peak numbers referring to Figs. 3 and 4. GR-7 and GR-21, 7- and 21-day granulomas respectively; ETC, embryonic-chick tendon cells; MPH, rat peritoneal macrophages.

Discussion

Relation of the present results to earlier work

Several authors (Evans, 1970; Barber & Jamieson, 1971; Perdue *et al.*, 1971; Lelievre, 1973) have described different subfractions of the plasma membranes from various tissues. Wray & Perdue (1974) obtained two plasma-membrane fractions from cultured fibroblasts with densities of 1.06 and 1.12, which correspond well to those reported above. The present 'light'

plasma membranes seem to contain additional non-membranous lipoprotein, perhaps as a contaminant, which accounts for the low purification factor.

The activities of 5'-nucleotidase and Na⁺,K⁺-activated Mg²⁺-dependent adenosine triphosphatase in granuloma preparations are higher than those reported for baby-hamster kidney cells (Gahmberg & Simons, 1970; Graham, 1972) or for chick-embryo fibroblasts (Bingham & Burke, 1972; Smith & Crittenden, 1973). On the other hand, in rat liver plasma membranes the activity of 5'-nucleotidase is about ten times that observed above for granuloma cells (Evans, 1970; Solyom *et al.*, 1972), as also in rat kidney (Price *et al.*, 1972).

The activities of the marker enzymes are not infallible criteria of the purity or yield. They may decline during the preparation of the membranes, and the actual yields may have been even better than indicated above. The marker enzymes occur in other cell parts too, and there are isoenzymes with variable activities (Lauter *et al.*, 1972).

Plasma membranes from granuloma compared with those from other cells with special reference to macrophages

Neville & Glossmann (1971) concluded that in the plasma membranes from liver, kidney brush-border and erythrocytes there was a common protein of about 48000 mol.wt., suggested to be actin-like (Wickus & Robbins, 1973). It is also present in the protein patterns shown in Figs. 3 and 5. The proteins of plasma membranes from granulation tissue and cultured fibroblasts (as reported by Greenberg & Glick, 1972) resemble each other over the molecular-weight range 30000-76000, but the plasma membranes from the cultured cells contain more high-molecular-weight material.

The cellular heterogeneity constitutes a common difficulty in assessing the results on plasma membranes prepared from solid tissues (cf. Wallach & Lin, 1973). In granulation tissue the macrophages may be

suspected as a source of contamination of the plasma membranes. However, Table 4 shows that in the plasma membranes of the peritoneal rat macrophages 5'-nucleotidase and leucine β -naphthylamidase are more active than in those from granulation tissue, whereas the reverse is observed for Na^+, K^+ -activated Mg^{2+} -dependent adenosine triphosphatase. The gel-electrophoretic distributions of proteins were also clearly different (Fig. 5).

This evidence does not support a significant contamination of the plasma membranes from the fibroblasts by those from macrophages. The plasma membranes from granulation tissue were prepared in the absence of EDTA, when the plasma membranes from macrophages aggregate and do not sediment in the same sucrose densities as those from the fibroblasts.

For work on the metabolism of the connective tissue the presence of macrophage-derived membranes is not critical. The present plasma-membrane preparations from the granulation tissue did not contain enriched activities of either collagen proline hydroxylase or lysine hydroxylase or collagen glycosyltransferases (J. Risteli & K. Kivirikko, personal communication).

The plasma membranes from the granulation tissue seem to differ also from those of the tendon cells. The tendon cells are more differentiated. There is no information on the types of collagen in granulation tissue, but the cross-linking differs from that of skin collagen (Bailey *et al.*, 1973; Jackson *et al.*, 1974).

Comparison of various developmental phases of granulation tissue

The yield of plasma membranes was lower but the purification was better for proliferating than for mature granuloma (Table 2), which may be due to a easier fragmentation of the plasma membrane. Another difference was seen in the phospholipid/protein ratio (Table 3), perhaps because proportionally more protein molecules appear in the lipid phase (cf. Singer & Nicolson, 1972) during the course of the development. The specific activities of the marker enzymes decreased to about one-half of those in the mature, 21-day phase, if calculated in terms of protein (Table 4). When the calculation was based on total phospholipids of the plasma membrane, some difference still persisted between the developmental phases.

The plasma membranes from proliferating tissue contain the protein peaks 14 and 15, which are small in plasma membranes from more mature tissue (Figs. 3 and 5). Also, in plasma membranes from mature tissue as compared with those from proliferating granuloma, the peaks 2-5 (proteins with mol.wt. 76000-160000) stain more intensively when the main peaks 7-9 (mol.wt. 40000-56000) are taken as the

respective references ($n = 5-7$). In the glycoprotein patterns (Fig. 4) there is a difference in peak 3 (mol.wt. 117000) which stains as a glycoprotein first in the mature phase.

The data in Table 4 on the activities of the various marker enzymes or the protein patterns in Fig. 5 are not consistent with the suggestion that the differences between 7-day and 21-day granulation tissue would depend on a varying proportion of fibroblasts and macrophages.

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