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ISOLATION AND CHARACTERIZATION OF SURFACE MEMBRANES FROM CHORIOALLANTOIC CELLS AND CHICK FIBROBLASTS

ALAIN ISRAEL, MARIE-ANGE VERJUS and MARIANNE SEMMEL

Laboratoire de Biologie Moléculaire (Dr J. Huppert), Institut Gustave-Roussy, Villejuif (94800), (France) (Groupe de Recherche No. 8 du C.N.R.S.)

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SUMMARY

Surface membranes were prepared from chorioallantoic cells and cultured chick fibroblasts by different methods. Mechanical disruption of cells followed by centrifugation through an aqueous two phase system and a sucrose gradient yielded a fraction composed mostly of surface membranes (enzymatic markers, cholesterol/protein ratio, phospholipid/protein ratio, electron microscopy). Results obtained by this method were fairly reproducible. Use of the method of Tremblay (sarkosyl-Mg crystals) yielded a mixture of cellular membranes, with great variations between experiments.

INTRODUCTION

Numerous methods for the isolation of surface membranes from eukaryote cells have been described[1,2,3]. Isolation procedures are based principally on the characteristic density properties of surface membranes, emphasized after binding of divalent cations, and on the characteristic chemical composition of these membranes. Efficiency of isolation, in terms of yield and purity of the final product depend on the original material and on the isolation procedure used. The isolation procedure may affect the properties of the isolated membranes[4,5]. Moreover, procedures and chemicals used during isolation may affect enzymatic and chemical assays used to characterize the products of different steps of purification[4,6].

In order to compare properties of surface membranes derived from chorioallantoic cells and chick fibroblasts we had to adjust existing methods so as to obtain a reasonable yield of purified membranes and to perform a series of characterization assays. The results will be described in this report.

MATERIALS

Cells

Chorioallantantoids were taken from 11-day-old embryonated lymphomatose free eggs and either used immediately or incubated up to 48 h at 37 °C in Eagles medium supplemented with 10% tryptose phosphate broth and 10% calf serum[7] with permanent stirring. Chick fibroblasts were cultured on Falcon plastic bottles in Eagles

medium supplemented with 10% tryptose phosphate broth and 10% calf serum. Secondary cultures were used for experiments. When cells were to be labelled with [5-^{3}H]uridine, tryptose phosphate broth was omitted from the medium.

Chemicals

Tris, glucose 6-phosphate, cytochrome *c*, NADH, and adenosine monophosphate were purchased from Sigma; EDTA and histidine from Fluka; ammonium molybdate, H_2SO_4, chloroform and cholesterol from Carlo Erba; serum albumin from Armour Pharmaceutical Company; polyethyleneglycol 6000 from BLB; dextran T 500 from Pharmacia; sarkosyl N 30 from Ciba, Geigy; 0.1 μm VCWP filters from Millipore; [5-^{3}H]uridine from C.E.A., Saclay. All other chemicals were Merck purest grade.

Equipments

Absorbance was measured with a Zeiss spectrophotometer PMQ II and radioactivity with a Beckman scintillation counter. For ultracentrifugations, an ultracentrifuge Spinco (Models L 50 or L 4 and rotors SW 39 or SW 65) was used. Electron micrographs were made with a Philips EM 300 electron microscope.

METHODS

An aqueous two-phase system (polyethyleneglycol–dextran) was prepared according to Brunette and Till[8]. Continuous gradients with an overlay of sarkosyl-Mg crystals were prepared according to Tremblay *et al.*[9]. Discontinuous sucrose gradients were prepared by layering sucrose solutions (65, 55, 45, 35 and 25% sucrose in imidazole–HCl buffer, 10^{-3} M, pH 7.2, w/w) over the particulate suspension in 85% sucrose in imidazole–HCl buffer[10].

Phospholipids were extracted with a chloroform–methanol mixture[11]. P_i was extracted from phospholipids according to Bartlett[12].

Proteins were determined by the method of Lowry *et al.*[13] or by absorbance measurement[14], cholesterol by the method of Glick *et al.*[15] and P_i by the method of Chen *et al.*[16].

5'-Nucleotidase was assayed using the method of Bosmann and Pike[17]. The reaction was modified to permit the determination of small quantities of 5'-nucleotidase: 0.1 ml cell suspension was incubated with 0.1 ml 4 mM AMP and 0.2 ml 1M Tris, $6 \cdot 10^{-2}$ M $MgCl_2$, pH 7.5. The reaction was stopped with 0.1 ml 40% trichloroacetic acid, the precipitate spun down and the supernatant used for the determination of P.

Glucose-6-phosphatase was assayed according to Swanson[18]. This method was also adapted to permit the detection of small amounts of the enzyme: 0.1 ml of suspension was incubated with 0.25 ml glucose 6-phosphate $8 \cdot 10^{-2}$ M, pH 6.5, 0.05 ml EDTA 10^{-2} M, pH 6.5, and 0.1 ml histidine–HCl buffer 10^{-1} M, pH 6.5. The reaction was stopped with 0.1 ml 40% trichloroacetic acid.

NADH-cytochrome *c* reductase was determined as described by Levy *et al.*[19].

For electron microscopy, an aliquot of the suspension was filtered through a Millipore filter VCWP, pore diameter 0.1 μm[20]. The filter was fixed for 1 h in 6% glutaraldehyde, washed in phosphate buffer 10^{-1} M, pH 7.4, fixed in 4% OsO_4, washed

in the same buffer, dehydrated and embedded in Epon. Thin sections were stained with 1% uranyl acetate and lead citrate and stabilized with carbon.

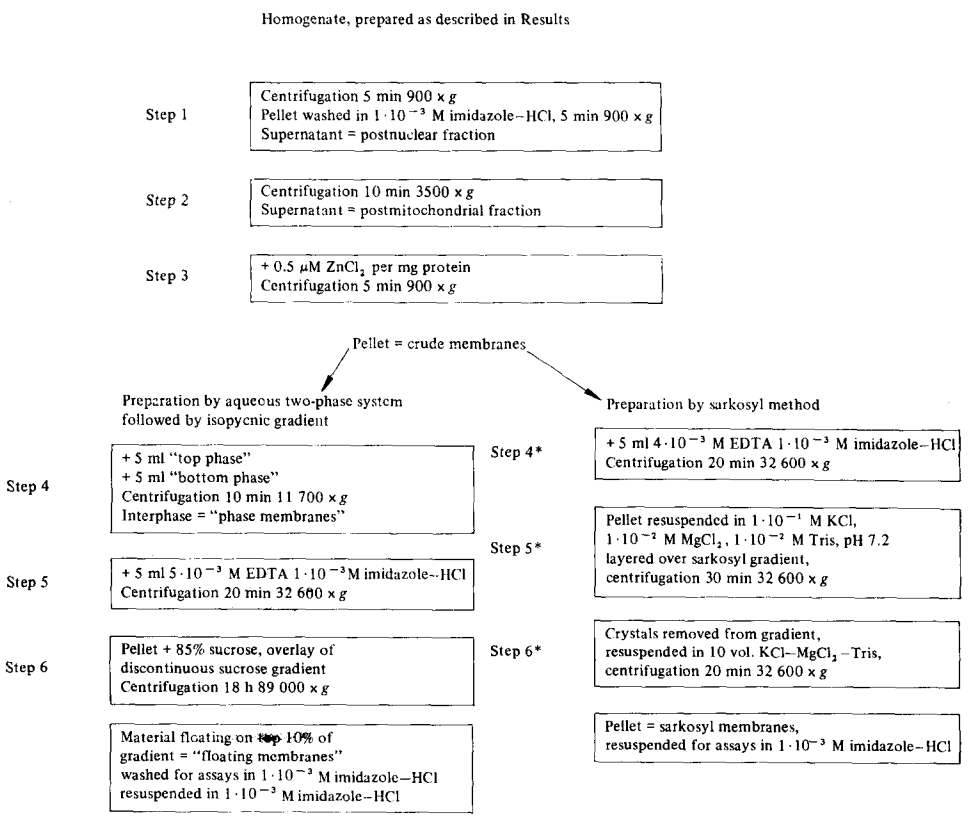
RESULTS

Isolation of surface membranes

2–5 chorioallantoids ($2 \cdot 10^8$–$5 \cdot 10^8$ cells)[21] were washed repeatedly in $1.4 \cdot 10^{-1}$ M NaCl, cut finely with bistouries, washed once in imidazole–HCl buffer, 10^{-3} M, pH 7.2 and sedimented for 5 min at $300 \times g$. The pellet was suspended in imidazole–HCl, kept for at least 45 min at 0 °C and homogenized with a Thomas homogenizer. This and subsequent steps were monitored by phase contrast microscopy. When 95% of the cells were disrupted (35–70 strokes were necessary), the suspension was filtered through four layers of gauze. The resulting suspension was called homogenate.

Monolayers of chick fibroblasts (about $5 \cdot 10^8$ cells) were washed with $1.4 \cdot 10^{-1}$ M NaCl, the cells removed from the plastic with a rubber tipped glass rod, washed once with imidazole–HCl and sedimented for 5 min at $300 \times g$. The pellet was resuspended in imidazole–HCl, kept for at least 45 min at 0 °C and homogenized with a Thomas homogenizer (about 25 strokes were necessary to disrupt 95% of the cells). The resulting suspension was called homogenate.

Subsequent steps were the same for both tissues and are illustrated in Scheme I.



The homogenate was centrifuged at low speed to sediment nuclei and unbroken cells. The supernatant was centrifuged at $3500 \times g$ to remove mitochondria and cellular debris. The protein content of the supernatant was determined by absorbance[14] and $ZnCl_2$ was added to precipitate membranes. The resulting crude membranes were treated with EDTA to remove Zn^{2+} and purified either by centrifugation through an aqueous two phase system[8] followed by an isopycnic gradient, or centrifuged through a sarkosyl Mg gradient[9]. The isopycnic gradient yielded, besides floating membranes, two bands of material at the 35–45% sucrose boundary and at the 45–55% sucrose boundary. The sarkosyl gradient showed, besides sarkosyl crystals, a band at the top of the gradient and a pellet. None of these components could be identified as membranes.

Several other methods were tried[10], but not further investigated either because the yield was very low[10] or because the method of preparation interfered with characterization assays[4,6].

In order to homogenize fractions for assays, Triton X-100 at a concentration of 0.17% was added. This detergent does not interfere with the activity of the enzymes assayed. Before assays, preparations were stored at 0 °C for at most 18 h.

Characterization of membranes

(*a*) *Sarkosyl.* Sarkosyl has been used to isolate membrane bound DNA and RNA[9,22,23]. The method was originally developed for bacterial cells[9] and later used for eukaryote cells. Characteristics of the membranes thus isolated were not determined[22,23]. Recently, reports of the isolation with sarkosyl of nexuses and gap junctions from mammalian cells have been published[24,25].

Table I summarizes some characteristics of membranes of chick fibroblasts and chorioallantoic cells prepared by this method and sedimenting with sarkosyl crystals. Wide variations of both yield and purity occurred between experiments, whether chorioallantoic cells or chick fibroblasts were used. Protein yield was relatively high, but there was no or little enrichment in cholesterol. More 5'-nucleotidase activity (membrane marker[17]) was found in the membrane fraction than in the homogenate. However, in some preparation sglucose-6-phosphatase activity (endoplasmic reticulum marker[18]) was as much or more concentrated as was 5'-nucleotidase activity. (Sarkosyl, up to a concentration of 0.2% does not interfere with these enzyme activities.) When cells were labeled for 4 h with [5-^{3}H]uridine and the radioactivity/mg protein of both homogenate and membrane fraction were compared, an increase of the specific radioactivity of the membrane fraction was observed (by a factor of 2.2 for chorioallantoic cells and a factor of 1.1 for chick fibroblasts).

Figs 1b and 1c show an electron micrograph of material sedimenting with sarkosyl crystals. Besides membrane fragments, partially broken mitochondria (mi) and agglomerates, presumably altered ribosomes and denatured proteins, can be observed.

Both the electron micrographs and the results of assays show that these preparations contain many components other than surface membranes. Moreover, the variations between experiments and the relatively low yield of cholesterol indicate that this method is unsuitable for the preparation of pure surface membranes from chorioallantoic cells and chick fibroblasts.

(b) *Two-phase system.* A rapid method of preparation of surface membranes

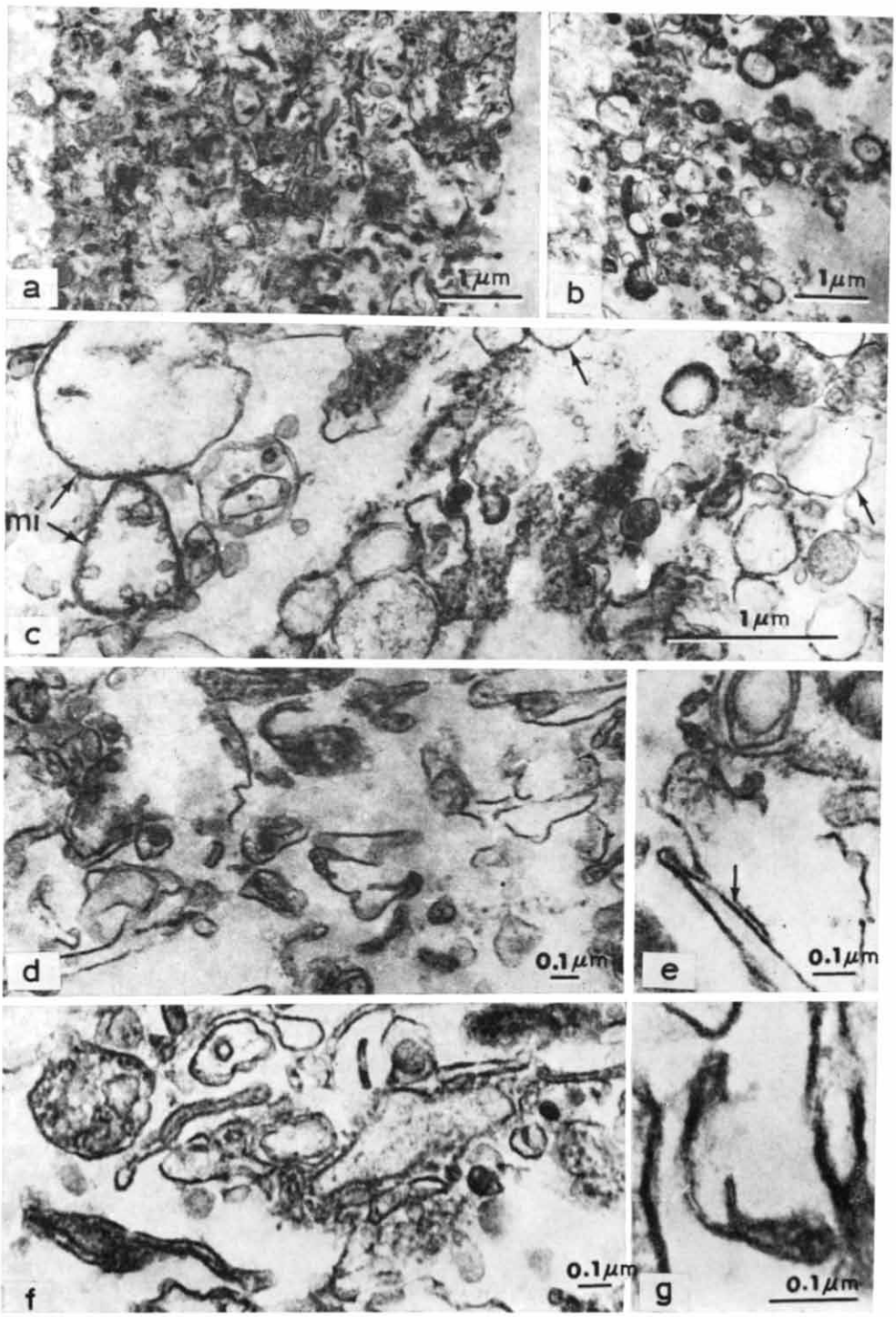
TABLE I

CHARACTERIZATION OF MEMBRANES ISOLATED WITH SARKOSYL (MATERIAL SEDIMENTING WITH SARKOSYL CRYSTALS)

Percentages are calculated from homogenate (100%). t.l.d.: too low to be detected; n.d.: not determined.

	Chorioallantoic cells											
Experiment No.	*1*		*2*		*3*		*4*		*5*		*Mean*	
	%		%		%		%		%		%	Average deviation
Yield protein (μg)	3.0	(1716)	1.3	(550)	0.8	(709)	3.1	(2960)	1.1	(714)	1.8	± 0.9
Yield cholesterol (μg)	5.0	(221)	2.3	(89)	4.3	(220)	6.8	(400)	2.7	(48)	4.2	± 1.0
Relative cholesterol content (μg/mg protein)	166	(128)	176	(161)	537	(310)	219	(135)	245	(67)	268	±107
Activity 5′-nucleotidase (nmoles P/60 min/mg protein)	180	(219)	161	(168)	159	(286)	390	(168)	250	(200)	228	± 73
Activity glucose-6-phosphatase (nmoles P/30 min/mg protein)	188	(284)	t.l.d.	(t.l.d.)	207	(255)	n.d.	(n.d.)	380	(264)	155	±116

	Fibroblasts											
Experiment No.	*1*		*2*		*3*		*4*		*5*		*Mean*	
	%		%		%		%		%		%	Average deviation
Yield protein (μg)	3.0	(536)	2.9	(310)	0.5	(189)	10.0	(934)	0.6	(221)	3.4	± 2.6
Yield cholesterol (μg)	9.4	(50)	5.4	(35)	2.9	(128)	1.5	(118)	1.8	(99)	4.2	± 2.5
Relative cholesterol content (μg/mg protein)	313	(93)	186	(113)	580	(677)	15	(126)	300	(448)	278	±142
Activity 5′-nucleotidase (nmoles P/60 min/mg protein)	340	(430)	180	(106)	780	(222)	440	(782)	830	(871)	514	±232
Activity glucose-6-phosphatase (nmoles P/30 min/mg protein)	170	(398)	t.l.d.	(t.l.d.)	240	(243)	150	(241)	980	(1278)	308	±268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from L cells by centrifugation through an aqueous two-phase system has been described[8]. Cell homogenate is precipitated with $ZnCl_2$, the precipitate is resuspended in an aqueous two-phase system (polyethyleneglycol–dextran) and centrifuged for 10 min at $10700 \times g$. The material sedimenting at the interphase represents a crude membrane fraction. When chorioallantoic cells and chick fibroblasts were used instead of L cells and a postmitochondrial fraction instead of cell homogenate, the material sedimenting at the interphase yielded a relatively high amount of both protein and cholesterol. Concentration of cholesterol, 5′-nucleotidase activity and glucose-6-phosphatase activity were higher in these fractions than in homogenate (Table II). Specific radioactivity of RNA decreased (by a factor of 0.88 in chorioallantoic cells and by a factor of 0.84 in chick fibroblasts).

Electron micrographs (Fig. 1a) show that material sedimenting at the interphase contains beside membrane fragments, many agglomerates, presumably denatured proteins, but no identifiable organelles.

These results show that surface membranes can be isolated from chorioallantoic cells and chick fibroblasts by this method, but that further purification is needed to obtain preparations reasonably free of endoplasmic reticulum and other contaminants.

(c) *Purification in an isopycnic gradient.* When the material sedimenting at the interphase of the aqueous two phase system was resuspended in 85% sucrose and spun in an isopycnic gradient prepared as described in Methods[10] the topmost 10% of the gradient contained membrane fragments and few other components. Electron micrographs (Figs 1d and 1e for chorioallantoic cells and Figs 1f and 1g for chick fibroblasts) show a material composed of more or less vesiculous membrane fragments. Some foamy material, which may be discrete cytoplasmic material, is often found adsorbed on the vesicles. The structure of these membranes is clearly visible on Figs 1e and 1g. Characteristics of the material obtained in the top 10% of an isopycnic gradient are summarized in Table III.

When chorioallantoic cells were used, different experiments yielded comparative results. When fibroblasts were used, variations between experiments were somewhat greater. Compared to the homogenate, the preparations were enriched in cholesterol, phospholipids and 5′-nucleotidase activity, whereas the activities of glucose-6-phosphatase and NADH cytochrome *c* reductase (this enzyme is found mostly in mitochondria but does occur also in endoplasmic reticulum)[19,20] decreased. Specific radioactivity of RNA decreased also (by a factor of 0.5 for chorioallantoic cells and by a factor of 0.13 for chick fibroblasts).

Fig. 1. (a) Two-phase system. The Millipore surface is at the left of the figure. The important deposit of material contains many membrane fragments and granular agglomerates which can be identified as more or less denatured proteins and ribosomes ($\times 12000$). (b) Sarkosyl method: same representation of the material collected on the Millipore filter as in (a) ($\times 12000$). (c) Sarkosyl method: a magnification of another zone shows that the granular aggregates are less abundant than in two-phase system prepared membranes. Some altered mitochondria (mi) can be recognized with their residual cristae and their characteristic double membrane. Many circular membraneous profiles are present (→) $\times 25000$. (d) Chorioallantoic cell plasma membranes obtained by the isopycnic gradient technique ($\times 50000$). (e) The trilamellar structure of the membrane is indicated by an arrow ($\times 65000$). (f) Same preparation as in (d) and (e), but from chick fibroblasts ($\times 50000$). (g) The fine structure of the membrane is clearly visible ($\times 130000$).

TABLE II

CHARACTERIZATION OF MEMBRANES PREPARED BY CENTRIFUGATION THROUGH AN AQUEOUS TWO-PHASE SYSTEM (MATERIAL SEDIMENTING AT INTERPHASE)

Percentages are calculated from homogenate (100%). n.d.: not determined.

	Chorioallantoic cells											
Experiment No.	*1*		*2*		*3*		*4*		*5*		*Mean*	
	%		%		%		%		%		%	Average deviation
Yield protein (μg)	1.4	(3450)	5.6	(4464)	3.3	(3230)	7.6	(7370)	8.0	(4897)	5.2	± 2.2
Yield cholesterol (μg)	7.2	(220)	10.3	(522)	n.d.	(n.d.)	11.4	(617)	21.9	(390)	12.7	± 3.1
Relative cholesterol content (μg/mg protein)	514	(64)	183	(117)	n.d.	(n.d.)	150	(91)	273	(80)	280	±117
Activity 5′-nucleotidase (nmoles P/60 min/mg protein)	230	(57)	229	(411)	310	(116)	70	(299)	340	(270)	235	± 70
Activity glucose-6-phosphatase (nmoles P/30 min/mg protein)	320	(56)	180	(230)	140	(115)	290	(256)	260	(185)	239	± 61

	Fibroblasts											
Experiment No.	*1*		*2*		*3*		*4*		*5*		*Mean*	
	%		%		%		%		%		%	Average deviation
Yield protein (μg)	3.2	(814)	7.8	(3530)	13.3	(2757)	8.0	(3020)	10.0	(6600)	8.4	± 2.5
Yield cholesterol (μg)	10.0	(53)	11.0	(74)	2.5	(71)	n.d.	(n.d.)	29.1	(432)	13.1	± 7.9
Relative cholesterol content (μg/mg protein)	312	(65)	141	(21)	19	(26)	n.d.	(n.d.)	291	(65)	190	±110
Activity 5′-nucleotidase (nmoles P/60 min/mg protein)	110	(132)	110	(67)	n.d.	(n.d.)	270	(133)	198	(148)	172	± 62
Activity glucose-6-phosphatase (nmoles P/30 min/mg protein)	180	(422)	300	(427)	180	(132)	150	(222)	245	(169)	211	± 49

TABLE III

CHARACTERIZATION OF MEMBRANES FLOATING ON A DISCONTINUOUS SUCROSE GRADIENT (TOPMOST 10%)

Percentages are calculated from homogenate (100%). t.l.d.: too low to be detected; n.d.: not determined.

	Chorioallantoic cells											
Experiment No.	*1*		*2*		*3*		*4*		*5*		*Mean*	
	%		%		%		%		%		%	Average deviation
Yield protein (μg)	0.4	(220)	0.5	(422)	0.3	(340)	0.8	(804)	0.3	(218)	0.46	± 0.15
Yield cholesterol (μg)	3.7	(142)	3.6	(181)	n.d.	(n.d.)	9.3	(548)	4.1	(72)	5.17	± 2.06
Relative cholesterol content (μg/mg protein)	925	(645)	720	(428)	n.d.	(n.d.)	1162	(681)	1366	(330)	1043	±220
Activity 5′-nucleotidase (nmoles P/60 min/mg protein)	580	(607)	240	(424)	410	(217)	1050	(452)	620	(500)	580	±184
Activity glucose-6-phosphatase (nmoles P/30 min/mg protein)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	15	(13)	t.l.d.	(t.l.d.)	3	± 5
Activity NADH-cytochrome *c*-reductase (units $A_{550\ nm}$/min/mg protein)	n.d.	(n.d.)	n.d.	(n.d.)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	t.l.d.	
											nmoles P/mg protein	
Relative phospholipid content* (nmoles P/mg protein)		(0.22)		(0.21)		(0.22)		(0.21)		(0.25)	0.22	± 0.04
											μg/nmoles P	
Cholesterol/phospholipid (μg/nmoles P)*		(0.86)		(0.99)		(0.87)		(0.97)		(0.71)	0.88	± 0.08

TABLE III (*continued*)

Experiment No.	*Fibroblasts* 1		2		3		4		5		*Mean*	
	%		%		%		%		%		%	Average deviation
Yield protein (μg)	0.9	(328)	0.7	(470)	0.6	(370)	1.1	(180)	3.3	(90)	1.3	± 0.7
Yield cholesterol (μg)	n.d.	(n.d.)	3.6	(81)	3.8	(56)	6.0	(60)	3.1	(40)	4.1	± 0.9
Relative cholesterol content (μg/mg protein)	n.d.	(n.d.)	514	(172)	633	(151)	545	(333)	94	(444)	446	±176
Activity 5′-nucleotidase (nmoles P/60 min/mg protein)	133	(270)	550	(378)	480	(328)	300	(141)	370	(44)	394	±124
Activity glucose-6-phosphatase (nmoles P/30 min/mg protein)	t.l.d.	(t.l.d.)	280	(280)	120	(89)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	80	± 96
Activity NADH–cytochrome *c*-reductase (units $A_{550\ nm}$/min/mg protein)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	t.l.d.	(t.l.d.)	n.d.	(n.d.)	n.d.	(n.d.)	t.l.d.	
											nmoles P/mg protein	
Relative phospholipid content* (nmoles P/mg protein)		(0.74)		(0.73)		(0.77)		(0.79)		(0.75)	0.76 ± 0.03	
											μg/nmoles P	
Cholesterol/phospholipid (μg/nmoles P)*		(0.136)		(0.136)		(0.111)		(0.111)		(0.115)	0.122 ± 0.014	

* To determine the ratios μg cholesterol/nmoles P phospholipids and nmoles P phospholipids/mg protein a separate series of experiments was done, as there was not enough material to do all assays with a single preparation.

DISCUSSION

Chorioallantoic cells comprise both fibroblasts and epithelial cells and constitute a cohesive tissue resistant to mild methods of homogenization. Cultured chick embryo cells contain more than 90% fibroblasts. However, the physiological state of cells may differ in one culture as well as in different cultures, mainly as a function of cell density. These cells are broken more easily.

The properties of proteins, in particular enzyme activities, are maintained when cells are homogenized by mechanical means and a reduction of osmotic pressure. Isolation and purification of surface membranes by mild procedures such as centrifugation through an aqueous two-phase system[7] or a sucrose gradient[10] permits the conservation of these properties up to the final product. $ZnCl_2$ inhibits some enzyme activities, for instance 5′-nucleotidase activity. As this cation was used for the concentration of suspensions and in the two-phase system, it was necessary to remove it by EDTA before enzyme assays. At the low concentrations used, EDTA does not interfere with membrane configuration.

Membrane preparations obtained by a combination of the methods of Brunette and Till[8] and Perdue and Snyder[10] could be compared in many respects both with each other, with the homogenate and with the products of intermediate steps of purification. The morphology of the membrane fractions thus purified did not differ whether chorioallantoic cells or chick fibroblasts were used: differences of biochemical characteristics between different preparations are in the range of those described in the literature[8,21].

Membranes isolated by centrifugation through a two phase system comprise many more components other than surface membranes, in particular endoplasmic reticulum and unidentifiable material.

When membranes were prepared with sarkosyl[9], values varied widely between experiments, presumably because the ratio of membrane fragments of different origin and size to sarkosyl-Mg crystals varied. Results indicated that the preparations contained a mixture of cellular membranes. Therefore, this method seems unsuitable for the preparation of pure surface membranes, It might, however, be used for the rapid preparation of a mixture of cellular membranes from different types of cells.

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