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CELL SURFACE CONSTITUENTS OF SARCOMA 180 ASCITES TUMOR CELLS*

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SUMMARY

The cell surface protein components of Sarcoma 180 ascites tumor cells have been investigated by a combination of plasma membrane isolation techniques and lactoperoxidase iodination. For plasma membrane isolation cells were homogenized in the presence or absence of Zn^{2+} and fractionated by sucrose density gradient centrifugation or a two-phase partition to give large membrane fragments or membrane envelopes. Membrane purification was monitored by phase contrast microscopy and chemical and enzyme marker assays. The membrane preparations were analyzed by acrylamide gel electrophoresis in sodium dodecylsulfate. Each preparation showed a common protein pattern of about 15 bands ranging in molecular weights from 33000 to >300000. Two carbohydrate-containing bands were also present in all preparations. Membranes prepared with Zn^{2+} were much less fragmented and showed much greater amounts of three high molecular weight components than those prepared in the absence of Zn^{2+}. This might suggest a role for these components in membrane stabilization.

The tumor cells were also subjected to iodination with lactoperoxidase, followed by membrane isolation and acrylamide gel electrophoresis in sodium dodecylsulfate in order to identify polypeptides accessible to the cell surface. The major radioactive band coincided with the major carbohydrate-containing band, presumably a surface glycoprotein. A second carbohydrate-containing band showed variable labeling behavior between different cell preparations. This material had a high molecular weight, as indicated by both acrylamide gel electrophoresis and gel permeation chromatography in dodecylsulfate. Several other components are labeled to a lesser extent in the intact cell.

INTRODUCTION

Plasma membranes are a complex mixture of proteins, glycoproteins, neutral lipids, phospholipids and glycolipids, possibly with some polysaccharide attached. Although particular plasma membrane functions must be dependent on membrane component organizations and their organizational changes, very little is known

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Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; INT, 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium.

about molecular details of plasma membrane structure. Considerable progress has been made in delineating some features of erythrocyte membrane structure[1–5], but many questions still remain. For more complex cells the problem is aggravated by difficulties inherent in the isolation of plasma membranes without intracellular membrane contamination[6], which makes even an enumeration and quantitation of plasma membrane components difficult.

In the present work we have concentrated on describing the major proteins and glycoproteins associated with plasma membranes prepared by several techniques, all of which involve isolation of large membrane fragments. In addition lactoperoxidase iodination, which has been used successfully with other mammalian cells[7–11], has been investigated as a means of assessing which of these components are accessible to the cell surface.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free ^{125}I was purchased as NaI from New England Nuclear. Lactoperoxidase, isolated from bovine milk[12], was a gift of Dr K. E. Ebner.

Growth and isolation of Sarcoma 180 ascites tumor cells

Sarcoma 180 tumor cells were maintained by weekly intraperitoneal injection of approx. $1 \cdot 10^5$ cells in 0.2 ml of 0.9% NaCl into COBS albino mice (HaM/ICR) of both sexes. After 6–8 days approx. $8 \cdot 10^7$ cells were recovered from the peritoneal cavity of a mouse. Ascites cells were collected by aspiration and centrifuged ($70 \times g$, 3 min). Cells were washed twice with isotonic saline before use for isolation of subcellular fractions. Only ascites preparations containing a minimal number of erythrocytes were used for further experimentation.

Plasma membrane isolation by the procedure of Atkinson and Summers[13]

Washed tumor cells were suspended in 15 vol. of 10 mM Tris (pH 8.0) for 2 min and centrifuged ($600 \times g$, 2 min). This procedure hemolyzes erythrocytes and leaves ghosts in the supernatant. The pellet of swollen tumor cells was suspended in 10 vol. of 10 mM Tris and homogenized by five strokes of a Dounce homogenizer with a tight pestle. Immediately after homogenization the suspension was brought to a concentration of 3 mM in Mg^{2+} by the addition of 30 mM $MgCl_2$–100 mM NaCl. The homogenate was then centrifuged at $1000 \times g$ for 1 min and the pellet (nuclear fraction) was washed once with 3 mM $MgCl_2$–10 mM Tris–10 mM NaCl. The pooled supernatants were centrifuged at $10\,000 \times g$ for 10 min to yield a pellet (crude plasma membrane) and the supernatant fraction containing soluble protein and small particles. A 5-ml sample of the pellet in 10 mM Tris was layered onto a sucrose density gradient of 30 ml of 30% (w/w) sucrose–10 mM Tris over 10 ml of 45% sucrose and centrifuged in an SW 25.2 head at $10\,000 \times g$ for 20 min. The bands at the interfaces, in the 30% layer and at the bottom of the tube were collected, washed twice with 10 mM Tris and used for phase contrast microscopy and chemical and enzymatic assays.

Plasma membrane isolation by the procedure of Warren and Glick[14]

Washed, swollen tumor cells were suspended in 10 vol. of 2 mM $ZnCl_2$ at 25 °C

for 10 min and at 0 °C for another 20 min. The cells were homogenized with a tight pestle (10–20 strokes) until most (> 70%) cells appeared ruptured, as ascertained by phase contrast microscopy. The cells were diluted with 10 vol. of 40 mM Tris (pH 7.4) and centrifuged at 3000 × *g* for 3 min. The pellet was washed once with 40 mM Tris. The washes were combined (supernatant fraction). The washed pellet (crude plasma membrane, containing nuclei) was layered onto a sucrose gradient of 10 ml each of 35, 40, 45 and 55% (w/w) sucrose and centrifuged in an SW 25.2 head at 27000 × *g* for 1 h. Bands at the 35–40% (W-SDG-II) and 40–45% (W-SDG-III) interfaces contained plasma membrane envelopes free of nuclei with little background particulate material. These were washed with 40 mM Tris and used for analyses.

Plasma membrane isolation by the two-phase method of Brunette and Till[15]

Tumor cells were treated with 5 vol. of 1 mM $ZnCl_2$, homogenized and diluted with 3 vol. of 1 mM $ZnCl_2$. The suspension was centrifuged at 1000 × *g* for 10 min and washed once with 3 vol. of $ZnCl_2$ solution. The pellet was suspended in three times the original cell volume of upper phase prepared by the method of Brunette and Till[15], and an equal volume of bottom phase was added. After mixing thoroughly, the phases were separated by centrifugation at 10000 × *g* for 10 min in an SW 25.1 rotor. The two phases and the interface material were decanted from the pellet of whole cells and nuclei and recentrifuged. Material at the interface was collected and washed two times with 40 mM Tris. The collected membranes were used for phase contrast microscopy, enzyme assays and electrophoresis.

Membranes (2.3 mg protein/ml) prepared by this method were treated with 10 mM EDTA in 40 mM Tris at pH 7.4 for 1 h at 0 °C. Treated and untreated membrane samples were subjected to discontinuous sucrose density gradient centrifugation on a gradient of 10 ml each of 35, 40, 45 and 55% (w/w) sucrose for 1 h at 27000 × *g* in an SW 25.2 rotor.

Lactoperoxidase iodination of Sarcoma 180 cells

To 10 ml of washed ascites cell suspension ($3 \cdot 10^7$–$5 \cdot 10^7$ cells/ml) in Krebs–Ringer phosphate (pH 7.4) was added 5 ml of labeling mixture (10 μM KI, 100 μCi/ml $Na^{125}I$ and 0.5 μM lactoperoxidase in Krebs–Ringer solution). Aliquots of 50 μl of H_2O_2 solution (2.3 mM H_2O_2 in 0.9% NaCl–7 mM phosphate at pH 7.4) were added with shaking at 30-s intervals for 12 min at room temperature. The mixture was incubated an additional 5 min, and 0.5 ml of 100 mM KI was added. The cell suspension was centrifuged at 3000 × *g* for 4 min at 4 °C, and the cells were washed twice with 0.9% NaCl–7 mM phosphate. Cell fractions and plasma membranes were prepared after homogenization in the presence of Zn^{2+} by the two-phase method[15]. In control experiments lactoperoxidase or H_2O_2 was omitted from the reaction mixture.

For labeling of cell homogenates 3 ml of cell suspension ($7 \cdot 10^7$ cells/ml) in 0.9% NaCl–7 mM phosphate (pH 7.4) were mixed with 3 ml of 40 mM Tris (pH 7.4) with or without 1 mM $ZnCl_2$ and homogenized by 150 strokes in a tight Dounce homogenizer. Labeling mixture was added and iodination performed as described above. Aliquots of the resulting suspension were centrifuged at 2000 × *g* for 4 min and 50000 × *g* for 3 h to obtain low and high speed supernatant fractions, respectively. The remainder of the suspension was used to prepare plasma membranes by the two-phase method[15].

Lactoperoxidase iodination of isolated plasma membranes

Plasma membranes were isolated by the two-phase method and suspended in 40 mM Tris (pH 7.4) at 9 mg protein/ml. To 4 ml of membrane suspension was added 1.5 ml of labeling mixture (10 μM KI, 100 μCi/ml $Na^{125}I$, 0.5 μM lactoperoxidase in 40 mM Tris). Aliquots of 20 μl of H_2O_2 (2.3 mM in 40 mM Tris) were added at 30-s intervals at room temperature for 13 min and the labeling was terminated by adding 200 μl of 100 mM KI. The membranes were centrifuged and washed three times with 40 mM Tris.

Analytical procedures

Subcellular fractions were assayed for enzymatic activities without additional treatments. Fractions for chemical assays were dialyzed against distilled water at 4 °C overnight. Protein was determined by the method of Lowry *et al.*[16], and sialic acid by the method of Warren[17]. Phospholipid was extracted by the method of Folch *et al.*[18], and determined as phosphorus[19]. NADH diaphorase and ATPase were assayed by the procedures of Wallach and Kamat[20], except histidine–imidazole buffer and ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) were used in the ATPase assay instead of Tris and EDTA. Succinate 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium (INT)-reductase, 5′-nucleotidase and glucose-6-phosphatase were assayed as described by Morré[21].

Dialyzed, lyophilized samples for acrylamide electrophoresis were dissolved in 4% dodecylsulfate–2% mercaptoethanol–40 mM phosphate (pH 7.4) at a concentration of about 4 mg protein per ml and incubated at room temperature overnight. One-fourth volume of 50% glycerol–0.05 mg/ml pyronin Y–50 mM phosphate was added, and 100 μl of this mixture was applied to the top of 5% gel. Electrophoresis was performed for 4 h at 8 mA/gel. Gels were stained for protein and carbohydrate as previously described[22]. Appropriate quantities of washed membranes, supernatants (after dialysis to remove I^-) or gel slices[23] were digested with NCS solubilizer (Amersham/Searle) in counting vials at 50 °C for 3 h[24]. After addition of Bray's solution[25], the samples were counted on Packard Tri-Carb model 3320.

Electron microscopy

Membrane samples were fixed by a modification of the procedure of Karnovsky[26] for 2 h and washed three times with cacodylate buffer with an overnight suspension in the final wash buffer. The samples were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h and dehydrated by a graded series of ethanol washes. After washing in propylene oxide, the samples were embedded in D.E.R. 332–736 (Polysciences, Data sheet 113) and cured at 45 °C under vacuum. Sections were cut on Porter–Blum ultramicrotome and examined with a Phillips 200 electron microscope.

RESULTS

Plasma membrane isolation

Three different methods have been investigated for the isolation of plasma membranes from Sarcoma 180 ascites tumor cells, as described under Experimental Procedures. Membrane purification was monitored by phase contrast microscopy

and chemical and enzyme assays. Representative samples were examined by electron microscopy. The 5′-nucleotidase and glucose-6-phosphatase activities of these cells were quite low, and these enzymes could not be used reliably as enzyme markers.

The procedure of Atkinson and Summers[13] yielded quite fragmented membranes, which could be purified by sucrose density centrifugation. Acrylamide gel electrophoresis of homogenates and whole cells indicated that no significant proteolysis occurred during the homogenization process. Recoveries and specific activities for the various constituents are given in Tables I and II, respectively. The (Na^+, K^+)-ATPase from other preparations of membranes prepared by this procedure

TABLE I

RECOVERIES OF CHEMICAL CONSTITUENTS AND ENZYMES

Values are averages ±S.D. with the number of preparations in parentheses and are expressed as percentages of the amount in the cell homogenate. SDG, sucrose density gradient fraction. N.D., not determined.

Constituent	*Percent recovered*		
	Atkinson and Summers[13] procedure		*Warren and Glick[14] procedure*
	Crude plasma membrane	*SDG I+II*	*W-SDG II+III*
Protein	11.3± 3.1 (3)	2.0±0.5 (3)	4.2±0.9 (3)
Cholesterol	40.5±10.2 (4)	14.2±5.7 (4)	25.2±9.9 (3)
Sialic acid	43.7±23.3 (3)	11.3±2.5 (3)	16.0±3.3 (3)
Phospholipid phosphorus	37.0±12.7 (2)	8.7±3.6 (3)	15.7±5.3 (3)
NADH diaphorase	26.6±10.2 (3)	4.7±2.1 (3)	8.9±0.9 (3)
Succinate INT-reductase	N.D.	N.D.	3.7±1.1 (3)
Mg^{2+}-ATPase	27.9 (1)	17.5 (1)	49.6±4.2 (3)
(Na^+, K^+)-ATPase	57.9 (1)	21.5 (1)	39.6±1.4 (2)

TABLE II

SPECIFIC ACTIVITIES OF CHEMICAL CONSTITUENTS AND ENZYMES IN PLASMA MEMBRANE FRACTIONS ACCORDING TO ATKINSON AND SUMMERS[13]

Numbers in parentheses represent number of preparations used in obtaining average value. Chemical constituents are expressed as nmoles/mg protein, diaphorase as μmoles/min per mg protein and ATPase as μmoles/h per mg protein. SDG, sucrose density gradient fraction.

Constituent	*Homogenate*	*Crude plasma membrane*	*SDG I*	*SDG II*
Cholesterol	41.0 ±20.7 (3)	120.6 ±46.9 (3)	192.7 ±52.0 (3)	148.2 ± 29.3 (3)
Sialic acid	3.50 ± 1.91 (3)	17.2 ± 5.9 (3)	29.5 ±14.5 (3)	19.7 ± 9.5 (3)
Phospholipid phosphorus	94.7 ±15.1 (3)	323.1 ±52.7 (3)	406.7 ±95.6 (3)	356.1 ±150.1 (3)
NADH diaphorase	0.423± 0.10 (3)	1.06± 0.38 (3)	1.11± 0.26 (3)	0.84± 0.05 (3)
Mg^{2+}-ATPase	0.75 (1)	2.60 (1)	6.6 (1)	5.2 (1)
(Na^+, K^+)-ATPase	0.22 (1)	1.60 (1)	1.1 (1)	2.8 (1)

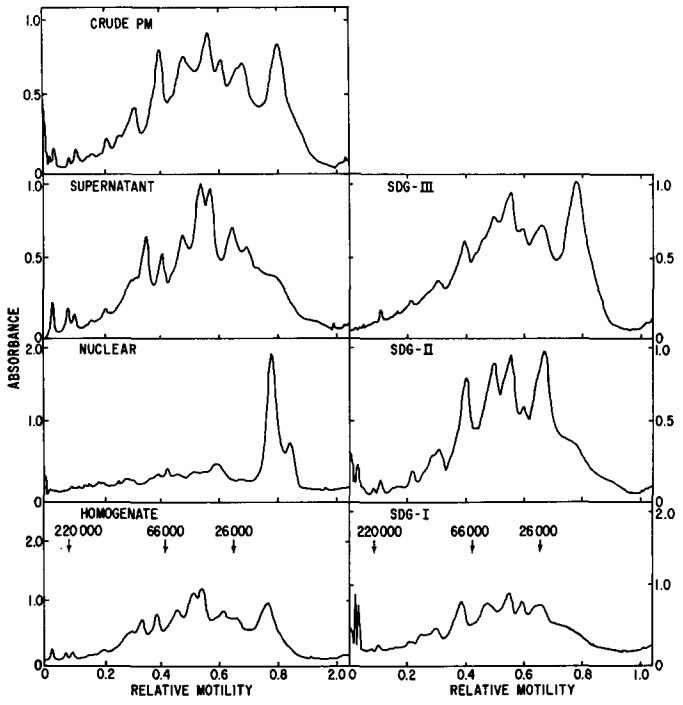

Fig. 1. Acrylamide gel electrophoresis profiles of fractions from Sarcoma 180 ascites tumor cells Details of preparation and electrophoresis are given in Experimental Procedures under the procedure of Atkinson and Summers[13].

showed approximately a 10-fold increase in activity over the homogenates (data not shown).

Samples at various stages of membrane purification were subjected to sodium dodecylsulfate electrophoresis. Profiles of gel scans of the various fractions are shown in Fig. 1. Comparisons of the profiles and of the gels themselves show the following features. The nuclear fraction contains predominantly proteins of lower molecular weight. Protein bands in plasma membrane fractions do not correspond to any of the major bands in the original homogenate except for the high molecular weight components, which are minor bands in terms of staining density in both fractions (homogenate or plasma membrane). This is to be expected, since the plasma membrane protein represents a small fraction of the total cellular protein. The crude plasma membrane is still contaminated by nuclei, as can be seen from both phase contrast microscopy and the acrylamide gel patterns. Virtually all of the nuclei in this fraction are recovered in Band IV of the sucrose density gradient centrifugation. Other than this nuclear contamination, there are few differences in the major staining components of the crude plasma membrane and the different fractions on the sucrose gradient. Only two bands were seen which stained significantly for carbohydrate by the periodate–Schiff stain procedure, as noted in a later section. The results from the acrylamide electrophoretic analyses were very similar throughout a series of several preparations from different batches of ascites cells.

The membranes prepared by the procedures of Warren and Glick[14] or Brunette and Till[15] are based on the "stabilization" of the cell surface membranes by Zn^{2+} before homogenization so that plasma membranes can be isolated as more nearly intact envelopes. The membranes are then purified by sucrose density gradient centrifugation for the former method and two-phase partition for the latter. Recoveries for the plasma membrane fractions prepared by the procedure of Warren and Glick[14] are given in Table I. The specific compositions and activities of these membranes were similar to those prepared by the method of Atkinson and Summers (Table II). The assays of plasma membrane markers indicated a purification of 5–7-fold for cholesterol, 3–5-fold for sialic acid and 5–20-fold for the Mg^{2+}- and (Na^+,K^+)-ATPases over the homogenate values. NADH diaphorase and succinate INT-reductase showed no change or slight decreases. Dodecylsulfate acrylamide electrophoretic analyses for the Warren and Glick membranes are shown in Fig. 2. The most noteworthy features of these analyses are the high molecular weight components present in the plasma membrane fractions which represent about 25% of the staining density. These

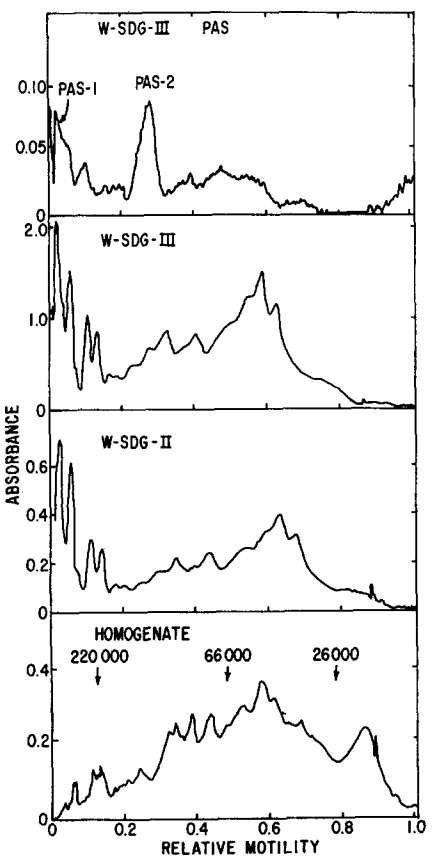

Fig. 2. Acrylamide gel electrophoresis profiles of carbohydrates (top) and proteins (bottom three) of plasma membranes and homogenate from Sarcoma 180 ascites tumor cells prepared by the method of Warren and Glick[14].

TABLE III

CONSTITUENTS OF MEMBRANE FRACTIONS PREPARED BY THE TWO-PHASE METHOD

Values are given for representative experiments and are expressed as in Table II, except for protein, which is given in percent. Fractions represent original homogenate, supernatant of homogenate and interface material from the second two-phase centrifugation.

Constituent	*Homogenate*	*Supernatant*	*Plasma membrane*
Protein	100	49.7	2.8
NADH diaphorase	0.37	0.32	0.25
Succinate INT-reductase	0.06	0.07	0.06
Mg^{2+}-ATPase	0.16	0.09	1.41
(Na^{+}, K^{+})-ATPase	0.12	0.0	1.01

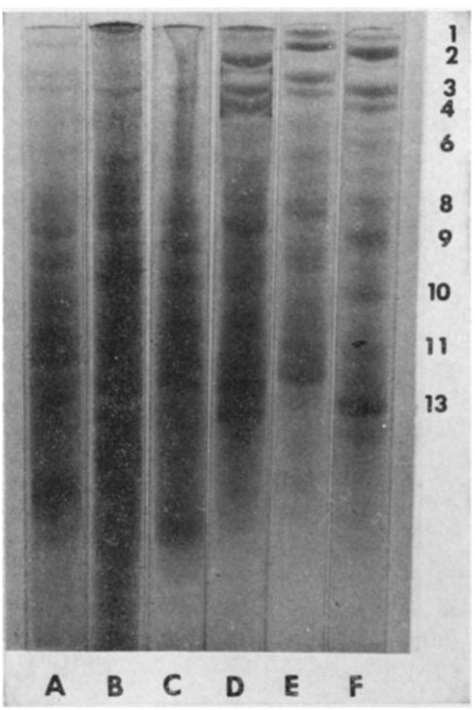

Fig. 3. Acrylamide gel electrophoresis of homogenates and plasma membrane fractions obtained by different preparative procedures. A, homogenate of the Atkinson and Summers[13] method; B, plasma membrane by that method; C, homogenate of the Warren and Glick[14] method; D, plasma membrane from the Warren and Glick[14] method; E and F, plasma membranes by the two-phase method[15]. Only the A and B, and C and D pairs are directly comparable, since the gels are from four different electrophoretic runs under slightly different conditions. The major bands in Gel F are numbered for identification. These same bands can be identified in Gels B and D on a band-by-band basis. Band 13 in Gel F does not show such a great staining intensity differential over the other bands around it in most preparations.

were not prominent components of plasma membranes prepared by the method of Atkinson and Summers[13]. The glycoproteins (periodate–Schiff-1 and -2) mentioned earlier are also shown in the top gel scan in Fig. 2. The electrophoretic profiles of membrane samples from different preparations were quite constant. Analytical data for membranes prepared by the two-phase method are shown in Table III. These are similar to results obtained with other two methods with the exception of the variation of the amount of ATPase activity present in the cells. The degree of purification of ATPase with the different membrane preparation techniques was more consistent than the amount of ATPase present.

The acrylamide electrophoresis patterns of membranes prepared by the two-phase procedure are shown in the photograph in Fig. 3 as Gels E and F. They can be compared to plasma membranes prepared by the other procedures (Gels B and D) and the original homogenates (Gels A and C). The different electrophoresis conditions for the different samples prevents direct comparisons, but comparisons can be made on a band-by-band basis. The membranes prepared by the two-phase system show a simpler profile with less background material. The major bands for the three types of preparations are the same except for the diminished amount of high molecular weight material on Gel B. There are some differences in band multiplicities and intensities between the different types of preparations. Only two major periodate–Schiff positive bands were demonstrated by all of the isolation methods. The bands in Gel F have been numbered for identification purposes. Table IV shows the molecular weights calculated for these polypeptides and an indication of their staining intensity for the

TABLE IV

MOLECULAR WEIGHTS OF MEMBRANE POLYPEPTIDES

Band number	*Molecular weight* ($\times 10^{-3}$)	*Relative staining intensity*		
		Atkinson and Summers[13] *procedure*	*Warren and Glick*[14] *procedure*	*Two-phase method*[15]
1*	>300	+	+	+
2*	280	+	+++	+++
3	237	+	+++	++
4	216	+	+++	++
5	190	+	+	+
6	178	+	+	+
7	148	+	+	+
8*	126	+	+	+
9	112	++	++	++
10	78	+++	++	+
11	56	+	++	++
12	48	+	++	++
13	42	+++	+++	+++
14	36	+++	+++	++
15	32	++	+	+

* Periodate–Schiff positive bands are present in the regions of Bands 1 and 2 and Band 8, but they may not correspond to these bands since glycoproteins often show poor staining characteristics with Coomassie blue[1].

various preparations. The two glycoproteins showed molecular weights of 126000 and >250000 on 5% gels, but these values are dubious because of the anomalous behavior of glycoproteins in the dodecylsulfate system[22].

The yields of protein and the presence of some succinate INT-reductase and NADH diaphorase indicate that the plasma membranes are still contaminated with some endoplasmic reticulum and mitochondria. Examination of sectioned membrane samples by electron microscopy showed no noteworthy differences from electron micrographs of similar preparations published previously[13,15]. The preparation by the method of Atkinson and Summers[13] consisted of membrane fragments and vesicles with some stained materials within the vesicles and adhering to the membranes. The preparation by the method of Brunette and Till[15] showed larger membrane sacs with some unstructured debris within the sacs. A considerable amount of stained material was also associated with the inside surface of the membranes. This might represent the high molecular weight material present only in the Zn^{2+} preparations, but we have no clear evidence of this. Both types of preparations showed only a few collapsed mitochondria and little or no rough endoplasmic reticulum.

Lactoperoxidase labeling of Sarcoma 180 cells

In order to assure that iodination was occurring at the cell surfaces of intact cells control experiments were performed without peroxide and lactoperoxidase (Table V) and on cell homogenates and isolated membranes (Table VI). Only 0.3% of the radioactivity in the incubation mixture was incorporated into the cells in the absence of lactoperoxidase, whereas 25% was incorporated in the presence of the enzyme. The pellet of the homogenate of the latter, which contained crude plasma membrane, unbroken cells and nuclei, had a 10-fold higher specific activity than the supernatant. Supernatant fractions were highly labeled in the cells which were labeled after homogenization, but the labeling of soluble constituents is minimal for labeled intact cells. Plasma membrane fractions show a 2–3-fold enhancement of specific activity in the intact cells over homogenized cells, and there is a 50-fold greater specific activity of plasma membranes than supernatant material (soluble and small particulate

TABLE V

REQUIREMENT FOR LACTOPEROXIDASE IN CELL LABELING

Cells were labeled as described in Experimental Procedures, washed twice with 0.9% NaCl–7 mM phosphate (pH 7.4) and homogenized. The homogenates were centrifuged 4 min at $3000 \times g$ to give a pellet and supernatant fraction, which were assayed for radioactivity and protein. ^{125}I incorporated is expressed as percentage of total ($5.4 \cdot 10^8$ cpm) in reaction mixture and specific activity as cpm/μg protein.

Labeling condition	*Fraction*	*^{125}I incorporated*	*Specific activity*
^{125}I only	Supernatant	0.12	2.7
	Pellet	0.20	1.8
$^{125}I + H_2O_2$	Supernatant	0.09	2.3
	Pellet	0.23	2.0
^{125}I, H_2O_2 and lactoperoxidase	Supernatant	0.8	19
	Pellet	23.8	208

TABLE VI

SPECIFIC ACTIVITIES OF SUBCELLULAR FRACTIONS OF LACTOPEROXIDASE-IODINATED CELLS AND PLASMA MEMBRANES

Intact cells, cells homogenized in the presence and absence of Zn^{2+} and isolated plasma membranes were iodinated with lactoperoxidase, ^{125}I and H_2O_2. Homogenates were centrifuged at $2000 \times g$ for 4 min (low speed supernatant) or $50000 \times g$ for 3 h (high speed supernatant). Plasma membranes were isolated by the method of Brunette and Till[15].

Labeling condition	*Fraction*	*Specific activity (cpm/μg protein)*
Intact cell*	Low speed supernatant	6
	High speed supernatant	6
	Plasma membrane	298
Homogenate, Zn^{2+}	Low speed supernatant	375
	High speed supernatant	374
	Plasma membrane	140
Homogenate	Low speed supernatant	537
	High speed supernatant	488
	Plasma membrane	108
Plasma membrane		4470

* The pellet of the low speed supernatant contains 97% of the bound radioactivity. The plasma membrane fraction contained 20% of the bound radioactivity and 0.9% of the cellular protein. The low yield of radioactivity is indicative of the low yields of plasma membranes obtained with these cells.

matter) in the intact cells. These results indicate that labeling occurs primarily at the plasma membrane in the intact cell. If lactoperoxidase labeling is occurring only at the cell surface of intact cells, one expects a selectivity of labeling of plasma membrane components as well as of subcellular fractions. This is demonstrated in Fig. 4, which shows plasma membranes iodinated as intact cells, homogenized cells and isolated plasma membranes. It is clear that the same proteins are not accessible in the intact cell and isolated membrane. This is to be expected if lactoperoxidase is not able to pass through the intact cell plasma membrane permeability barrier, since some proteins will be oriented toward the inside surface of the membrane, while others will be present at the exterior. Thus one would expect the fragmented isolated membranes to show an increased incorporation of iodine, which was found (Table VI). As another indication of the selectivity of labeling the radioactivity profile is compared to the staining pattern with Coomassie blue (Fig. 5). The positions of the two major periodate–Schiff positive bands are marked by arrows on the gel. It can be seen that the major peaks of radioactivity correspond to the positions of carbohydrate staining. This radioactivity profile, when compared to Fig. 4, also indicates the range of variability of labeling of the membrane components, particularly the band at the top of the gels.

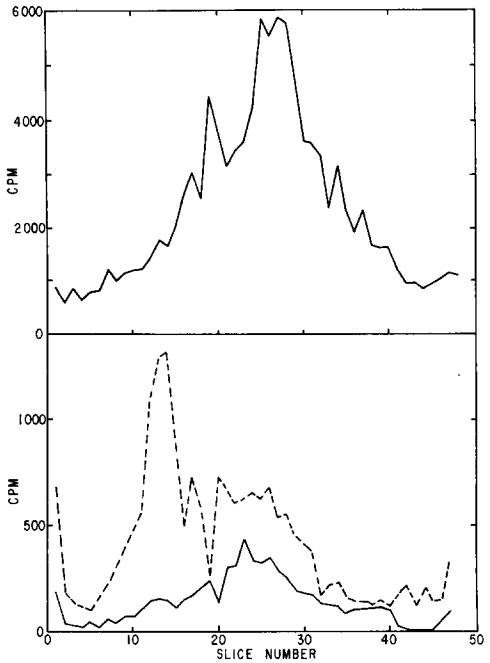

Fig. 4. Radioactivity profiles of plasma membranes from lactoperoxidase labeled Sarcoma 180 intact cells, homogenates and isolated plasma membranes. Isolated plasma membranes (top) were prepared by the two-phase method[15] before labeling. Whole cells (- - - - -) and homogenates (bottom, ———) were labeled as described in Experimental Procedures, and membranes were prepared from the labeled samples by the two-phase method.

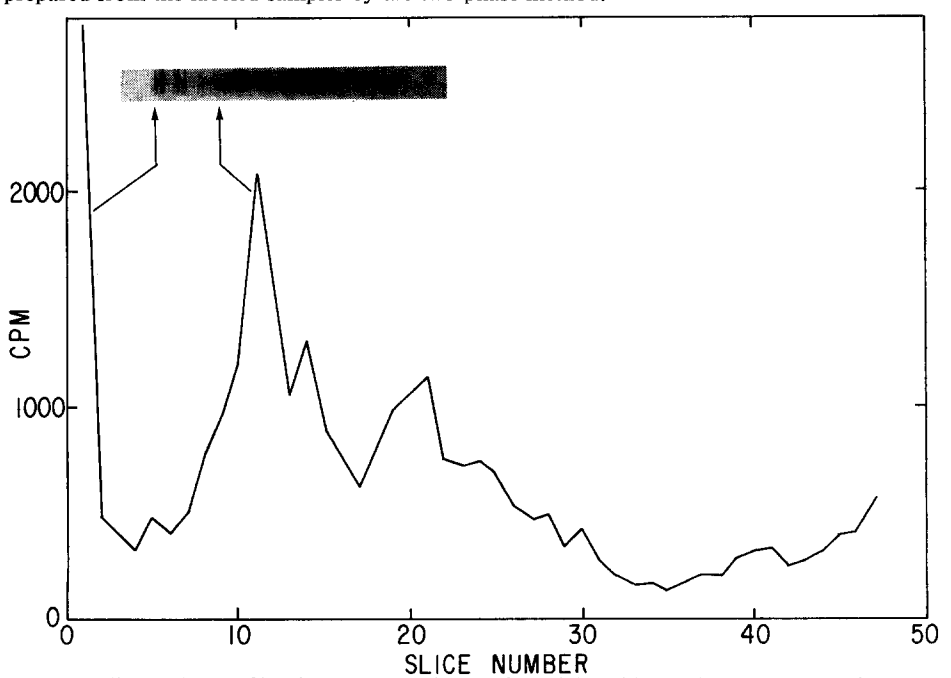

Fig. 5. Radioactivity profile of plasma membranes from labeled intact Sarcoma 180 ascites tumor cells. Photograph of gel stained with Coomassie blue is shown for membranes prepared from labeled cells.

DISCUSSION

Three methods of membrane isolation which yield morphologically identifiable membrane fragments were chosen for investigation. The use of Zn^{2+} in the homogenization medium does result in a greater degree of membrane integrity and permits isolation of larger fragments. In addition the Zn^{2+}-treated membranes have associated with the membranes large molecular weight polypeptides which are present in smaller amounts in the untreated and more fragmented membranes. An interesting speculation is that the high molecular weight polypeptides stabilize the membrane in the presence of Zn^{2+}. Support for this idea comes from the similarities of the polypeptides to spectrin of the erythrocyte membrane, which has a similar molecular weight, associates with the erythrocyte membrane and is aggregated in the presence of divalent cation[27].

With the exception of the high molecular weight polypeptides the proteins are quite similar for the three preparations. Also the extent of purification (about 10-fold) of the (Na^+, K^+)-ATPase, an enzyme commonly used as a plasma membrane marker, was similar for the three types of preparations. The use of Zn^{2+} to prevent membrane fragmentation clearly offers some advantages in identifying the membrane fragments, and the two-phase method appears to give less contaminating material (both by phase contrast microscopy and acrylamide electrophoresis). One disadvantage of all of these methods with these cells is the low yield, as judged by the membrane markers. This results in part from the variability of the susceptibility of the cells to disruption. One must compromise between mild homogenization, which leaves large numbers of undisrupted cells, and more vigorous homogenization, which fragments the membranes such that they are lost during isolation procedures.

The use of lactoperoxidase to label cell surfaces must be applied with care in complex cells to prevent misinterpretation of labeling results. At least three criteria should be met in order to establish reaction at the cell surface. (1) There should be minimal iodine incorporation in the absence of lactoperoxidase, since this might suggest the presence of intracellular enzymes capable of iodination which would not be limited to the cell surface. (2) Labeling should be limited to the plasma membrane fraction of the cell. Any labeling of soluble cellular components would suggest that the enzyme penetrated the plasma membrane permeability barrier. (3) There should be a different selectivity for components labeled in the intact cell plasma membrane and the isolated, fragmented plasma membrane. This follows from the asymmetric nature of the plasma membrane, in which different components are accessible at the exterior and interior surfaces of the membrane[1]. These criteria have been established with erythrocyte membranes and have been shown to hold fairly rigorously in the present study.

Labeling of intact Sarcoma 180 ascites tumor cells consistently shows the presence of one major labeled species. It corresponds to a periodate–Schiff positive band (presumably a glycoprotein) with an apparent molecular weight of 126000 on 5% acrylamide gels. The actual molecular weight is questionable in view of the known anomalous behavior of glycoproteins on dodecylsulfate electrophoresis[22]. A second periodate–Schiff positive band shows variable labeling behavior in different cell preparations. This material barely penetrates the 5% acrylamide gel in dodecylsulfate and is also excluded from Sepharose 4B, indicating a high molecular weight. The

unusual behavior of this material has not yet been explained. The labeling at the top of the gels does not appear to result from aggregation of other non-glycoprotein membrane proteins, since there is no enhancement of Coomassie blue staining at the tops of gels in samples that are more highly labeled. The periodate–Schiff staining and the iodination, taken together, suggest it is a glycoprotein, although the evidence is not conclusive. It will be necessary to isolate and characterize this material in order to understand its nature and relationship to the cell surface more clearly.

An additional interest in this component derives from the fact that Poduslo *et al.*[11], have shown the labeling of a similar high molecular weight component in baby hamster kidney and L cells. The distribution of the baby hamster kidney and L cell plasma membrane polypeptides on acrylamide electrophoresis[29] is quite similar to that of the Sarcoma 180 cells, except for the presence of the additional major glycoprotein in the Sarcoma cells. In the study of Poduslo *et al.*[11], the high molecular weight component was the only material that was significantly labeled, and it was shown to stain for carbohydrate. It was suggested that this material was a cell surface glycoprotein. These studies were complicated by a high degree of iodination in the absence of lactoperoxidase and by the fact that the high molecular weight material was the major labeled species in both the intact cells and isolated membranes, indicating that its enhanced labeling may be due to some factor other than its position at the exterior surface of the membrane. Examination of how these glycoproteins can exhibit different behaviors in different cell types or under different conditions may offer a clue to their involvement in cellular functions.

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