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ISOLATION AND CHARACTERIZATION OF THE PLASMA MEMBRANE OF L-1210 CELLS

IODINATION AS A MARKER FOR THE PLASMA MEMBRANE

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SUMMARY

Mouse leukemia L-1210 cells were iodinated with ^{125}I; this permitted the development of a method for the isolation of the plasma membranes. These show a 10- to 16-fold increase in the specific activity of ^{125}I over that of the cell homogenate and a 20-fold increase in the specific activities of 5'-nucleotidase and alkaline phosphatase; no mitochondrial or microsomal marker enzyme activities were detected. Sodium dodecyl sulfate gel electrophoresis of the plasma membranes shows approx. 40 peptides with molecular weights ranging from 10 000 to over 200 000; a polypeptide (M_r 50 000) predominates. Of 13 iodinated surface membrane proteins, the major radioactive peptide has a molecular weight of 85 000. The importance of the selection of the appropriate gel system for the analysis of membrane proteins is emphasized.

INTRODUCTION

Plasma membranes have been isolated by a variety of methods [1–5]. The recent reviews by Wallach and Lin [6] and by DePierre and Karnovsky [7] have emphasized two major difficulties: (1) to define a versatile plasma membrane marker and (2) to define a unique homogenization medium for cell disruption.

Our previous studies have shown that the Phillips-Morrison procedure [8] for the iodination of cell membranes in the presence of lactoperoxidase does not necessarily lead to the iodination of surface membrane proteins alone; it also labels internal membrane proteins as well as cytoplasmic proteins. We defined the conditions necessary for the iodination of surface membrane proteins alone [9, 10]. In the present investigation we have used optimal conditions to label the murine L-1210 cells, thus acquiring a marker for the plasma membrane which could be easily followed during the subsequent purification and characterization of the membranes.

Abbreviation: PMSF, phenylmethylsulfonylfluoride.

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MATERIALS AND METHODS

Radioactive ^{125}I was purchased from New England Nuclear. Lactoperoxidase was purified by the method of Morrison and Hultquist [11] through the CM-cellulose step of their purification procedure. Glucose oxidase and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma Co., Fischer's medium and horse serum from Grand Island Biological Co., pronase (B grade) from Calbiochem and hexyleneglyceol from Matheson Co. All other chemicals were of reagent grade.

Iodination procedure

L-1210 cells were grown in suspension culture in Fischer's medium supplemented with 10% horse serum at 37 °C in an atmosphere of O_2/CO_2 (95 : 5, v/v). Cells were harvested at the density of $5 \cdot 10^5$–$6 \cdot 10^5$ cells/ml, centrifuged at $250 \times g$ for 5 min and washed twice in Fischer's medium to remove the serum proteins. The cells were washed once more with 40 ml cold phosphate buffer (0.16 M, pH 7.0) per $5 \cdot 10^8 - 8 \cdot 10^8$ cells just before iodination. The iodination was carried out in 5 ml of iodination medium which contained $5 \cdot 10^8 - 8 \cdot 10^8$ cells, $5 \cdot 10^{-7}$ M lacteroperoxidase, 10 mM glucose, 5 munits glucose oxidase (assayed in 0.106 M phosphate buffer, pH 7.0, at 25 °C) and 50–100 μCi carrier-free $Na^{125}I$ in either pH 7.0 phosphate buffer (0.106 M) or in 0.25 M sucrose buffered with 10 mM phosphate, pH 7.0, at 2–4 °C for 15 min with shaking. The cells were then washed four times with Fischer's medium and once with phosphate buffer. At least 95% of the washed cells were viable as measured by trypan blue exclusion.

Radioactivity measurements

The acid-insoluble radioactivity was measured by a modification of the method of Mans and Novelli [12]. A portion of the sample was applied to a Whatman 3MM, 2.2 cm filter paper disc. The discs were washed in cold 10% trichloroacetic acid, 50 mM $K^{127}I$ for 1 h, transferred to cold 10% trichloroacetic acid for 30 min (repeat twice), rinsed with cold ether and then air dried. The radioactivity was determined by liquid scintillation counting in toluene/POPOP/Triton X-100.

Enzyme assays

(I) *5'-Nucleotidase.* 5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was measured by a modification of the method published by Chen et al. [13]. A 20–100-μl sample (either homogenate or membrane fraction) was added to 1 ml glycine buffer which contained 0.1 M glycine, pH 8.5, 10 mM $MgCl_2$, 5 mM 5'-AMP. After incubation at 37 °C for 15 min, 1 ml 10% trichloroacetic acid was added, the mixture was centrifuged at $1000 \times g$ and 1 ml supernatant was mixed with 3 ml 0.4 M sodium acetate to bring the pH to about 4; ammonium molybdate solution (0.3 ml, 1%, containing 1% ascorbic acid) was added to measure the inorganic phosphate released [14]. The absorbance was read at 700 nm after 20 min.

(II) *Alkaline phosphatase.* Alkaline phosphate (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity was measured by the method described in the Worthington manual [15] and the activity measured by following the increase in absorbance of nitrophenol at 410 nm.

(III) *Succinate dehydrogenase.* Succinate dehydrogenase (succinate-cytochrome

c reductase, EC 1.3.99.1) activity was measured by the increase in absorbance at 550 nm. A 20–50-μl sample was added to 1.2 ml phosphate buffer (20 mM phosphate at pH 7.0 with 1 % bovine serum albumin) at room temperature. Cytochrome *c* (1 %, 0.1 ml) was added and mixed. The substrate solution (50 mM succinate plus 10 mM KCN, 0.4 ml) was then added and mixed quickly and the absorbance at 550 nm was read immediately [16].

(IV) *NADPH - cytochrome c reductase* (*EC 1.6.2.3*). A 10–50-μl sample was added to 0.9 ml phosphate buffer (0.1 M phosphate, pH 7.4). Cytochrome *c* (1 %, 0.1 ml) was added and mixed. The reaction was initiated by addition of 0.1 ml 1 mM NADPH and the absorbance change at 550 nm was read. The activity of the enzyme was expressed as nmol cytochrome *c* reduced per min per mg protein [18].

(V) *Assay of cytochrome P-450.* A 50–100-μl sample was added to 1 ml phosphate buffer (0.1 M, pH 7.5, with 1 mM KCN) in the cuvette. The baseline was taken with an Aminco-chance dual wavelength spectrophotometer. Dithionite was added, the spectrum taken, CO bubbled for 20 s, and the spectrum taken again. The absorption difference in the region of 450 nm was taken to measure the amount of P-450 [17].

(VI) *Glucose-6-phosphate* (*EC 3.1.3.9*). A 50-μl sample was mixed with 0.3 ml maleic buffer (0.1 M, pH 6.5) and 0.1 ml of 0.1 M glucose 6-phosphate. The reaction was terminated by the addition of 1 ml cold 10 % trichloroacetic acid and centrifuged at 1500 rev./min for 5 min to remove the proteins. The inorganic phosphate in 1 ml supernatant was measured by the method described in 5′-nucleotidase assay [14].

Gel electrophoresis

The membranes were dissolved in 2–3 % sodium dodecyl sulfate with 5 % 2-mercaptoethanol and were immediately heated at 100 °C for 3 min. Cylinder gel electrophoresis was performed as described previously [9].

Sodium dodecyl sulfate gradient slab gel (7.5–15 %) was prepared and run according to the procedure of Alvares and Siekevitz [19] with the following modifications suggested by authors. Sucrose was added to the 7.5 % and 15 % acrylamide solution to a final concentration of 5 and 15 %, respectively, to stabilize the gradient; 5 % sucrose was also included in the stacking solution (5 % acrylamide). The gel thickness was reduced to 1.5 mm in order to dry the gel without cracking. The slab, after staining with Coomassie Blue, was dried onto Whatmann 3MM filter paper, and autoradiography performed using Kodak X-ray film as described by Maizel [20]. Slab gel electrophoresis was run at 150 V per slab for about 3 h until the bromophenol blue marker reached the bottom of the slab.

Electron microscopy

Electron microscopic examination of plasma membranes was performed after fixation in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The pellets were then transferred to 1 % OsO_4-containing cacodylate buffer (0.1 M, pH 7.4), dehydrated with ethanol and embedded in Vestopal-W [21].

RESULTS

Isolation of L-1210 plasma membranes

Step I. Disruption of cells. About 1 ml washed packed cells was suspended in 10 ml

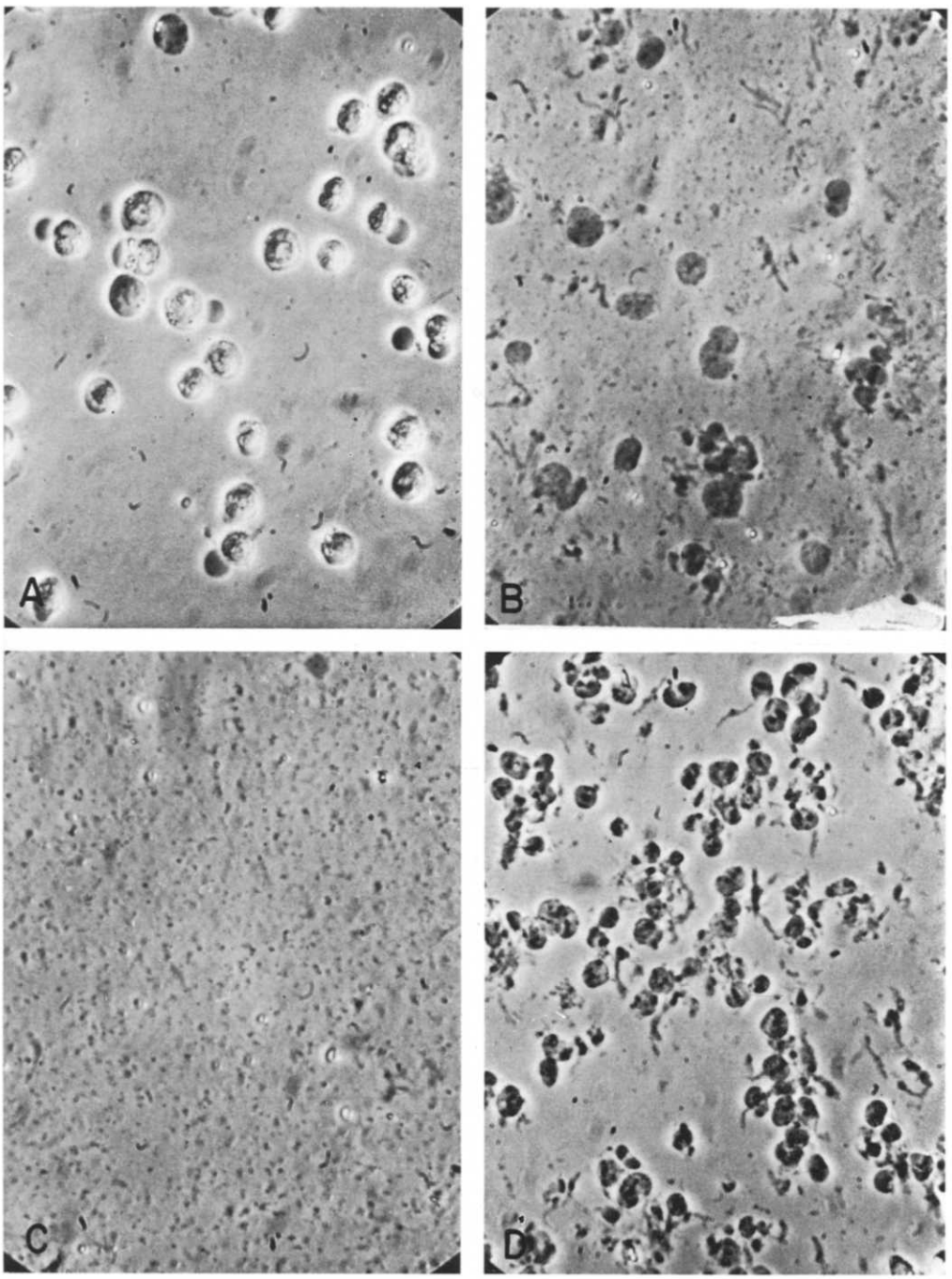

Fig. 1. Phase contrast microscopy ($\times 270$) of various fractions before and after cell rupture. (A), cell suspension in homogenization medium; (B), cell homogenate; (C), $1300 \times g$ supernatant; (D), $1300 \times g$ pellet.

of homogenizing medium (0.9 % saline, 10 mM sodium phosphate, pH 7.0, 0.5 M hexyleneglycol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM PMSF). The cells were ruptured by forcefully squirting 3-ml portions of the whole cell suspension through a 5-ml BD plastic syringe fitted with a 25G 5/8 inch needle against the bottom of scintillation vial with foaming. Approx. 9–10 strokes were needed to disrupt the cells as monitored by phase contrast microscopy in the presence of trypan blue; most of the nuclei remain intact. Phase microscopy of the cells before and after rupture is shown in Figs 1 A and 1 B. This fraction is designated as homogenate.

Step II. Removal of nuclei. The homogenate was centrifuged at $1300 \times g$ for 1 min, the pellet suspended in 4 vols of homogenizing medium and recentrifuged; the supernatants were combined, brought to 10 % sucrose by adding a 60 % sucrose solution and designated as supernatant fraction. The washed pellet and the supernatant fraction are shown in Figs 1C and 1D. The enrichment of a fraction in plasma membrane was determined by the ratio of the specific activity of the trichloroacetic acid-insoluble radioactivity of this fraction to that of the cell homogenate, i.e. the relative specific activity. Our experience indicates that in order to be able to finally obtain a

TABLE I

PURIFICATION OF L-1210 PLASMA MEMBRANES: DISTRIBUTION OF ^{125}I IN ALL FRACTIONS OF SUCROSE GRADIENT

The protein content is approx. 95 mg/10^9 cells. For description of the fractions, see Fig. 2.

Fraction	Volume (ml)	Trichloroacetic acid-insoluble (cpm/100 µl)	Protein (g/100 l)	cpm/µg	Relative specific activity	Radio-activity yield (%)	Protein yield (%)
Method I							
Homogenate	9.5	384 800	672	573	1.0	100	100
"Nuclei-free" homogenate	9.0	371 200	504	737	1.29	91	76
I-30	2.8	293 660	64.2	4570	8.0	22.5	3
I-30 supernatant	58	7 300	8.4	833	1.45	1.2	8.2
I-45	1.7	52 700	72	728	1.27	2.42	2.0
I-45 supernatant	63	300	0.8	400	0.7	0.5	0.84
TOP_1	22.4	42 060	99.2	421	0.73	25.72	37
30-45	39	6 860	5.5	1193	2.1	7.3	3.6
Bottom	3.1	73 900	118	626	1.1	6.32	6.2
II-30	5.6*	71 200	10.8	6593	11.5	10.9	1.0
II-30 supernatant	35	1 600	0.6	2667	4.65	1.5	0.35
II-45	3.9	24 120	6.4	3769	6.58	2.6	0.42
II-45 supernatant	13.3	200	0.7	300	0.5	0	0.16
Rest	8.4	2 370	6.0	3960	6.6	5.5	0.84
Method II							
Homogenate	14	31 965	741	43.2	1.0	100	100
"Nuclei-free" homogenate	13	29 893	632	47.3	1.1	86.8	79.3
I-30	3	39 725	125	318	7.4	26.6	3.6
C-30	1.0	60 600	101	600	13.9	14.4	1

* Volume before concentration.

plasma membrane fraction with a relative specific activity value above 10, the relative specific activity of the initial supernatant fraction (after removal of the nuclei) should be above 0.9.

Step III. Purification of the plasma membrane by sucrose gradient centrifugation. Method 1. This method included two discontinuous sucrose gradient centrifugations. The supernatant fraction containing 10 % sucrose was applied on a discontinuous gradient (7 ml 45 % sucrose, 14 ml 30 % sucrose) and centrifuged at 23 000 × g (swinging bucket, Sorvall HB-4 rotor, 12 000 rev./min) for 30 min. The 10–30 % interface layer was collected, diluted with saline (0.9 %) and pelleted by centrifugation at 33 000 × *g* for 30 min. This was designated as the I-30 fraction. The I-30 fraction was resuspended in saline and again applied on a second discontinuous gradient (3 ml 45 % sucrose, 5 ml 30 % sucrose and 3 ml 15 % sucrose) for 30 min at 23 000 × g. The 15–30 % interface fraction was collected, diluted with saline and pelleted. The pellet was resuspended in saline and designated as the II-30 membrane fraction. The II-30 fraction has a relative specific activity for ^{125}I of 10–16. The main characteristics of al-fractions obtained by this method are shown in Table I.

Method II. This method includes a separation on a continuous sucrose gradient. The I-30 fraction described above was diluted with 2 ml 0.9 % saline and centrifuged on a continuous sucrose gradient (9 ml from 60 to 30 % sucrose) at 140000 × *g*

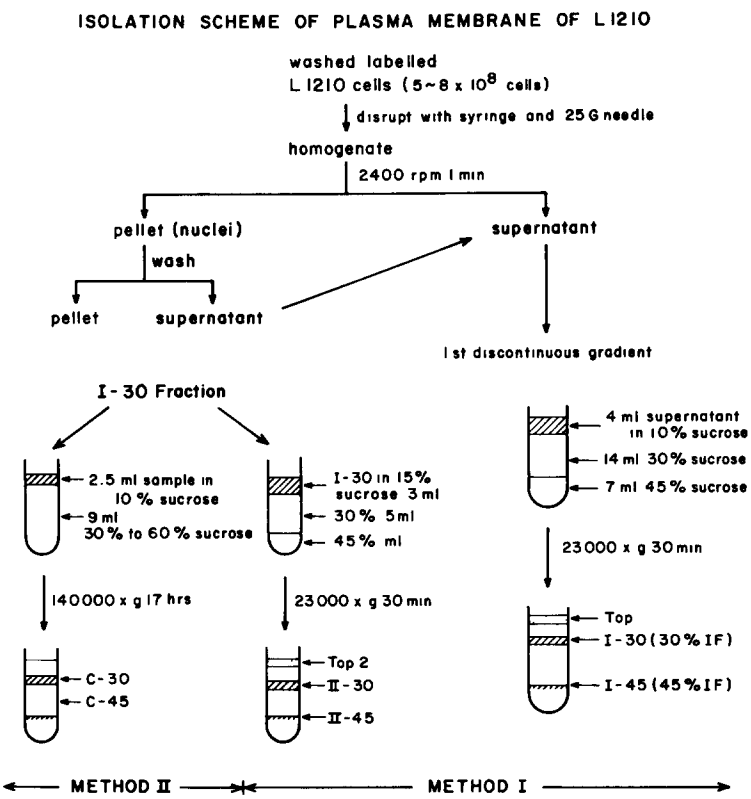

Fig. 2. Isolation scheme of mouse leukemic L-1210 cell membranes.

for 17 h. The opaque layer between 30 and 35 % sucrose was collected, pooled and pelleted after dilution with saline and centrifugation as above. The membrane pellet was suspended in saline and designated C-30. This gave a relative specific activity of 14 with a 14.4 % yield of radioactivity as compared to an 11 % yield of radioactivity for the comparable II-30 fraction. The isolation scheme is shown in Fig.2 and the relevant data are presented in Table I.

Purity of plasma membranes

Removal of the ^{125}I by proteolysis. As discussed in our previous papers [9, 10] pronase will digest a portion of the proteins on cell surfaces. Fig. 3 shows that two-thirds (66 %) of the acid-insoluble radioactivity could be removed by pronase. Under the same conditions, 70 % of the radioactivity was removed from iodinated HeLa cells [9]. Hubbard and Cohn [22] could remove 45 % of radioactivity from the iodinated red blood cells. The pronase digestion work supports the view that the lactoperoxidase-catalyzed iodination was limited to the cell surface in agreement with the autoradiographic work on lymphoma cells [23], platelet cells [24] and ascites cells [25] which showed that the labeled ^{125}I was limited to the cell surfaces.

Enzyme markers. Based on histochemical studies, plasma membranes locali-

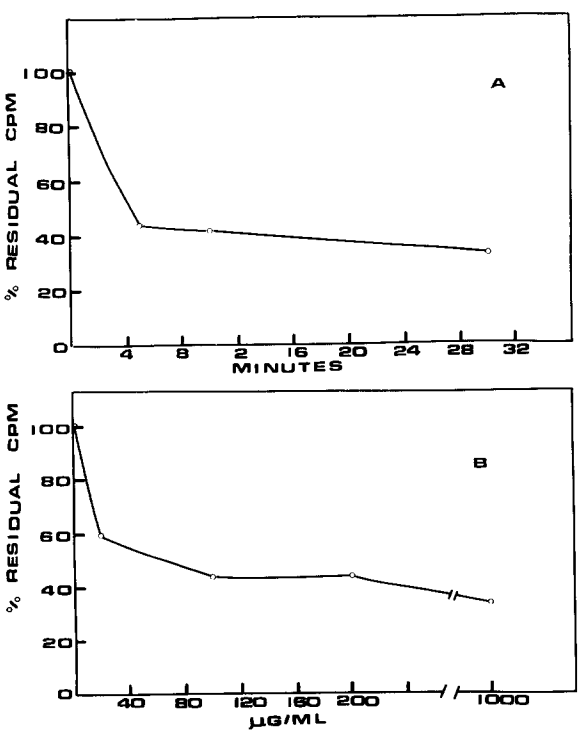

Fig. 3. Pronase digestion. Cells were iodinated as described in the iodination procedure and were then treated with pronase at 37 °C at cell concentration of $6 \cdot 10^6$ cells per ml. (A) The time course of the loss of acid-insoluble radioactivity from ^{125}I-labeled L-1210 cells upon treatment with 200 µg/ml pronase. (B) The effect of the concentration of pronase on the removal of ^{125}I-labeled proteins from L-1210 cells. The digestion time was kept at 10 min.

zation has been claimed for a number of enzymes, i.e. 5′-nucleotidase, ATPase, alkaline phosphatase, etc. [26]. Other specific organelle enzymes, for example, glucose 6-phosphate and P-450 for the endoplasmic reticulum, succinate dehydrogenase for mitochondria, aryl sulfatase for lysosomes and uridine diphosphatase for the Golgi apparatus [26], can also be used to determine the degree of contamination of the plasma membrane preparation. However, because of some intrinsic problems associated with the histochemical techniques and the broad spectrum of structure and functions of various cell lines, the enzymatic markers by themselves are not a direct evidence for the purity of plasma membrane. We have assayed six enzyme systems for the L-1210 cell line and the results are shown in Table II. Whenever the homogenate and the membrane fraction were used for enzymic assays, phenylmethylsulfonylfluoride was omitted from the homogenizing medium in the isolation procedure.

The two plasma membrane enzyme markers, 5′-nucleotidase and alkaline phosphatase show 20-fold increases in specific activities in our membrane preparations

TABLE II

n.d., not detectable.

	Specific activity (nmol/mg per min)	Relative specific activity	^{125}I-labeled relative specific activity
5′-Nucleotidase			
Homogenate	45.6	1	1
Membrane fraction	922	20	10
Alkaline phosphatase			
Homogenate	66	1	1
Membrane fraction	1383	21	9
Succinate dehydrogenase			
Homogenate	50 nmol cytochrome *c* reduced per min per mg	1	1
Membrane fraction	0	0	10
NADPH-cytochrome *c* reductase			
Homogenate	3.7 nmol cytochrome *c* reduced per min per mg	1	1
Membrane fraction	1.9	0.5	10
P-450			
Homogenate	n.d.		1
Membrane fraction	n.d.		10
Glucose-6-phosphatase			
Assay no. 1 in the absence of phenylphosphate			
Homogenate	2.7 ± 0.7	1	1
Membrane fraction	19.3 ± 6.8	7	10
Assay no. 2 in the presence of phenylphosphate			
Homogenate	0	0	1
Membrane fraction	0	0	9

when the relative specific activity for ^{125}I was enriched 9–10-fold. Time course studies for all the enzyme systems were done to ensure that the assays of enzyme activity were made within the linear range. Hinton [26] summarized the work up to 1972 on the isolation of liver plasma membranes; in this work, it is reported that the increase in the specific activity of 5′-nucleotidase in liver plasma membrane preparations ranged from 8- to 35-fold.

The mitochondrial marker enzyme, succinate dehydrogenase, was not measurable in our membrane preparation; this has been used as a good index of the absence of mitochondrial contamination [6, 7].

NADPH-cytochrome *c* reductase is a microsomal electron transfer enzyme: this activity for both the homogenate and the membrane preparation was found to be low in L-1210 cells. The NADPH-cytochrome *c* reductase activity which we measured is only 1–3 % of that of normal liver microsomal preparations. Another microsomal electron transfer enzyme, P-450, which is a marker for endoplasmic reticulum, was not detectable even in freshly prepared whole cells. In view of the absence of P-450 activity in L-1210 cells, the seemingly residual NADPH-cytochrome *c* reductase observed in the membranes might be due to non-specific reduction of the substrate.

Glucose-6-phosphatase has been generally considered to be a typical endoplasmic reticulum marker for the liver cells. However, it has also been suggested that it is present in the plasma membrane [27]. In our hands, the assay of glucose-6-phosphatase was further complicated by the fact that glucose 6-phosphate is a substrate for a number of phosphatases. Belfield and Goldberg [28] reported an enzyme "diversion" method to measure the activity of glucose-6-phosphatase in the presence of other non-specific phosphatases, namely, to measure glucose-6-phosphatase in the presence of excess of an alternate phosphatase substrate, disodium phenylphosphate; phenylphosphate at 10 mM concentration inhibited alkaline phosphatase, 5′-nucleotidase, and various non-specific phosphatases, but had little effect on glucose-6-phosphatase [29]. In Table II, the results of the assay of glucose-6-phosphatase in the absence as well as in the presence of disodium phenylphosphate are presented. In the absence of phenylphosphate, the plasma membrane fraction gave about a 7-fold increase of "glucose-6-phosphatase" activity, compared to the homogenate. In the presence of phenylphosphate, no glucose-6-phosphatase activity could be detected in either the homogenate or the membrane fractions. It therefore seems likely that the glucose-6-phosphatase activity that was measured in the absence of phenylphosphate was actually due to non-specific phosphatases. These results warrant that care be taken in using glucose 6-phosphate alone as a substrate for glucose-6-phosphatase activity.

Electron microscopy

Fig. 4 shows what appear to be reasonably clean vesicles, characteristic of plasma membranes, with very little contamination by other cell organelles.

Gel electrophoretic pattern of plasma membrane proteins

The gel radioactive patterns of plasma membrane proteins of L-1210 and HeLa cells are compared. Fig. 5A shows the radioactivity pattern after gel electrophoresis of cells [9]. The predominant radioactive proteins are in the high molecular weight region (M_r 100 000). However, the radioactivity pattern after gel electrophoresis of L-1210 cells was quite different, as shown in Fig. 5B; the broad radioactive band shows

Fig. 4. Electron micrograph of the isolated L-1210 plasma membranes, ×42 500.

a peak at about 80 000 daltons. Fig. 5C shows the radioactivity pattern obtained in 10 % acrylamide gel with 3.75 % cross-linking. Fig. 5D shows a considerably improved radioactivity pattern at an increased time of electrophoresis with 7.5 % gel, 3.75 % cross-linking. Using slab gel electrophoresis (7.5 % concentration, 2.7 % cross-linking) in a discontinuous buffer system [30], nine radioactive bands were obtained, as shown in Fig. 6B. The molecular weights of the radioactive bands were estimated to be 175 000, 165 000, 135 000, 118 000, 107 000, 105 000, 85 000, 70 000 and 50 000 ± 10 % with a major peak at 85 000. In a 7.5–15 % gradient slab gel, the smeared radioactive bands at 70 000 and 50 000 daltons in the 7.5 % slab gel became eight discrete bands (Fig. 7) and their molecular weights were estimated to be 75 000, 70 000, 63 000, 58 000, 50 000, 45 000, 37 000 and 13 000 ± 10 %. However, six radioactive bands above 85 000 daltons band in straight 7.5 % slab gel became four bands; two radioactive bands at molecular weights of 107 000 and 105 000 overlapped and appeared as a single fairly strong band at molecular weights 105 000; and the weak band at 118 000 daltons did not show up in the gradient slab gel. The radioactive band at molecular weights 13 000 runs off the gel in the straight 7.5 % slab gel sys-

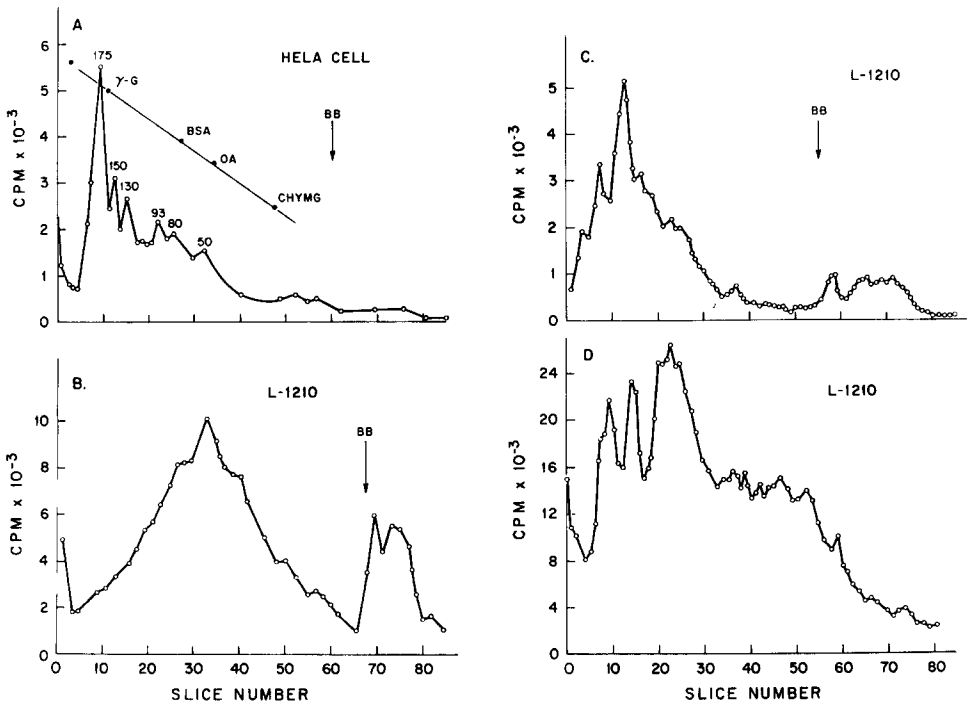

Fig. 5. The radioactive profile of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{125}I-labeled membrane proteins of L-1210 cells and HeLa cells. (A, B) 7.5 % gel, 1.5 % cross-linking, 10 mA/gel, 4 h, 0.1 M phosphate buffer, pH 7.0. BB, bromophenol blue. The numbers in indicate molecular weights in thousands. γ-G, human γ-globulin; BSA, bovine serum albumin; OA, ovalbumin; CHYMG, chymotrypsinogen A. (C) 10 % gel, 3.75 % cross-linking, 10 mA/gel, 4 h, 0.1 M phosphate buffer, pH 7.4. (D) 7.5 % gel, 3.75 % cross-linking, 10 mA/gel, 6.5 h, 0.1 M phosphate buffer, pH 7.4.

tem; this contains little protein when stained with Coomassie Blue as shown in Fig. 7A. The labeling patterns of the whole cells, supernatant fraction, and II-45 fraction were the same as that of plasma membranes (results are not shown). Among the above gel electrophoresis systems the gradient slab gel electrophoresis provided us the best discrete radioactive patterns while 7.5 % cylinder gel described in Figs 5A and 5B, which has a great resolving power for HeLa cell membranes, showed a broad radioactive peak with a molecular weight at 80 000 for L-1210 cells.

The protein stain of the cell homogenate and of the plasma membranes in 7.5 % slab gel (Fig. 6A) shows that more than 40 protein bands can be visualized in both fractions by Coomassie Blue, but their protein patterns are very different. The polypeptides of the plasma membranes are divided into four regions. Region I has 10 bands at least with molecular weight in the range 140 000–300 000; Region II has four easily visible bands plus 6–7 faint bands ranging from 90 000 daltons; Region IV, seven bands, from 25 000 to 65 000 daltons. There is a prominent peptide at molecular weight 50 000. In the gradient slab gel a similar protein stain pattern was observed as shown in Fig. 7A, and the proteins in the region smaller than the major 50 000 daltons peptide appeared to have better resolution. It is interesting to note that the plasma membrane has a major peptide with molecular weight 50 000, which has only trace

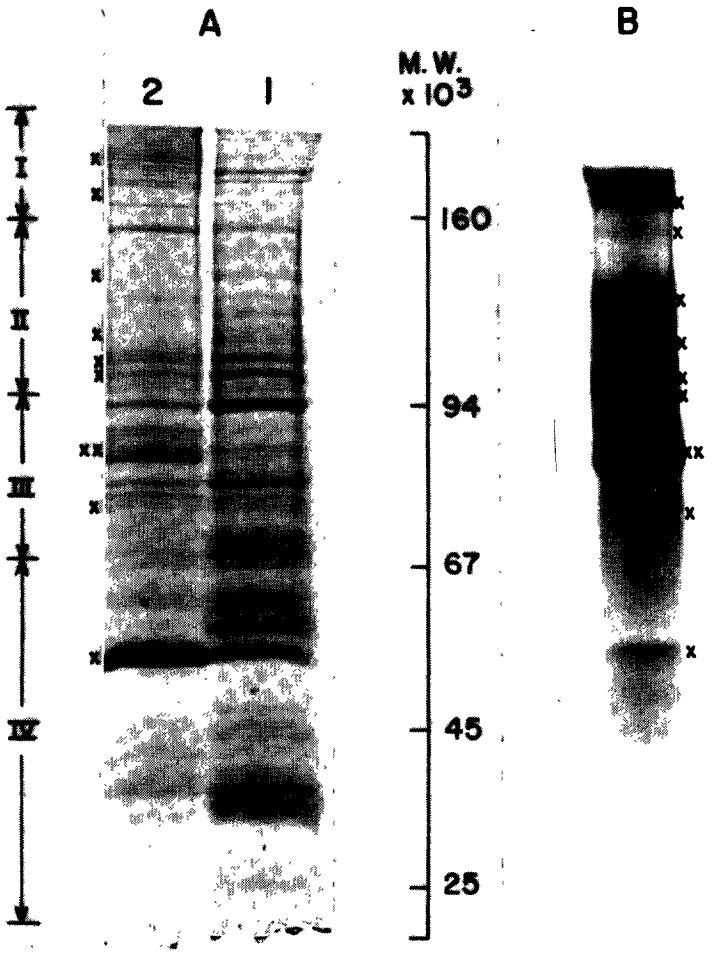

Fig. 6. The radioactive and protein profiles of the slab polyacrylamide gel electrophoresis of L-1210 cells in the presence of sodium dodecyl sulfate (7.5 % gel, 2.7 % cross-linking). (A) Coomassie Blue stain for protein of (1) cell homogenate and (2) plasma membranes (relative specific activity 13). The numbers on the right indicate molecular weights using human γ-globulin (160 000), phosphorylase a (94 000) bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 400) as markers. Cross marks in the middle show the location of radioactive bands revealed by autoradiography (B). xx indicates the major radioactive band.

radioactivity associated with this peptide. The amount of this peptide in the membrane fractions increases when the purity of membranes is improved and it shows up as the predominant peptide of purified plasma membranes on sodium dodycel sulfate acrylamide gel (Fig. 8).

Periodic acid-Schiff stain [31], after 7.5 % slab gel electrophoresis of plasma membranes, revealed three closely located glycoproteins at molecular weights of 96 000, 93 000 and 88 000 $\pm$ 10 % when 350 μg of membrane proteins were analyzed.

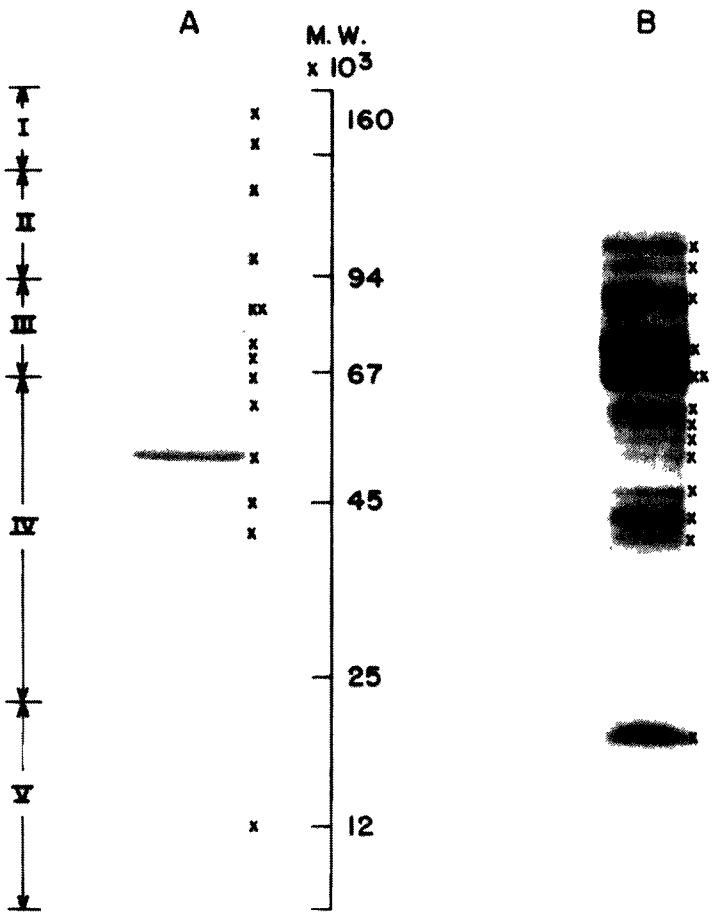

Fig. 7. The radioactive and protein profiles of the gradient slab gel electrophoresis of L-1210 membrane proteins in the presence of sodium dodecyl sulfate (a gradient of 7.5–15 % gel, 2.7 % cross-linking). (A) Coomassie Blue stain for proteins of L-1210 plasma membranes (RSA 9). (B) Autoradiography of the plasma membranes. The numbers and cross marks are described in Fig. 6.

DISCUSSION

We have established the optimal condition for the lactoperoxidase iodination of nucleated cells under which iodination occurs exclusively on cell surfaces [9, 10]. This permits ^{125}I to be used as a general plasma membrane marker and eliminates the uncertainty of the cellular location of an enzyme marker. Most investigators use marker enzymes, i.e. 5′-nucleotidase, ATPase, etc., to identify the plasma membrane during its isolation. However, the cellular location of an enzyme in one cell type may not be the same as that in other cell types, or in cells from different species. For example, 5′-nucleotidase is assumed to be present on the plasma membrane of mammalian liver cells, yet Wallach and Ullrey [32] reported that this enzyme is localized exclusively in the nuclei of Ehrlich ascites carcinoma cells. In addition it should be empha-

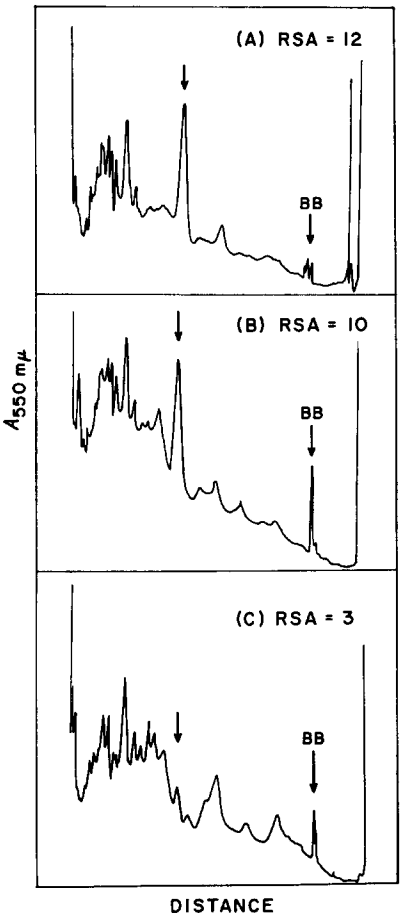

Fig. 8. Light scans of sodium dodecyl sulfate gels of membrane fractions with different relative specific activity values after staining with Coomassie Blue. Sodium dodecyl sulfate-polyacrylamide gels were prepared and run as described in Fig. 5C, the gels were then scanned at 550 nm after staining with Coomassie Blue. The arrow in the middle indicates the position of the 50 000 molecular weight peptide. BB, bromophenol blue.

sized that, in order to have an accurate assay of enzyme activity, the possible existence of enzyme inhibitors or activators which may be present in the crude homogenate should also be considered. The pseudoactivity of glucose-6-phosphatase in our preparation of plasma membranes is an example of the pitfalls inherent in such assays. In addition, the stability of an enzyme marker during the isolation procedure as well as the strength of its association with the membrane must also be considered. For these reasons we believe that our method of combining surface iodination with fractionation procedures has distinct advantages for the isolation of plasma membranes.

Once the plasma membrane has been isolated using this procedure, the increased specific activity of a certain enzyme in this fraction may indicate its in vivo association with the plasma membrane. Thus, in murine leukemia L-1210 cells, 5′-nucle-

otidase and alkaline phosphatase are found to be purified in parallel with the plasma membrane fraction. The differences in relative specific activities for these enzymes from that of ^{125}I may be an indication of inhibitors present in the crude homogenate. However, the exclusive location of these enzymes on the plasma membrane cannot be established and the possibility of the preferential attachment of cytoplasmic enzymes to the plasma membrane during isolation cannot be excluded.

The plasma membranes of L-cells [4], HeLa cells [33] and L-5178 Y cells [5] have been isolated in the form of vesicles or ghosts. From our experience, ghosts from L-1210 cells are difficult to obtain with good yield. Electron microscopy of the plasma membrane fraction of L-1210 cells shows reasonably clean membrane structures in the form of small vesicles as shown in Fig. 4. Larger vesicles were observed in the II-45 fraction (Table I) but these contained mitochondrial and other contaminants.

Recently, Hourani et al. [34] obtained crude plasma membranes of L-1210 cells using the two-phase polymer system of Brunette and Till [35]. Their procedure relies on the hypotonic swelling of L-1210 cells. However, Burger [36] reported that a lectin receptor complex was released from the cell membrane of L-1210 cell under sublethal hypotonic conditions. In order to avoid this loss, we have ruptured the cells in isotonic saline and at no time during the isolation was the membrane fraction subjected to hypotonic conditions. The labeling patterns of the whole cells and isolated plasma membranes are similar (results are not shown). This suggests that, using our technique, no loss of labeled plasma membrane components occurred during the isolation procedure.

Cell membrane preparations have also been obtained from L cells [35], cultured human lymphocytes [37] and Ehrlich ascites tumor cells [25] using the two-phase system of polyethyleneglycol and dextran. The specific activity of $(Na^+ + K^+)$-ATPase, presumably a plasma membrane marker enzyme, was increased 8-fold for both L-cells and L-1210 cells. Electron micrographs of the L-cell membrane fraction revealed large membrane fragments with particles attached to their surfaces [35]. By coupling the lactoperoxidase iodination technique with the two-phase polymer system mentioned above, membranes were isolated from Ehrlich ascites tumor cells [25], cultured human lymphocyte [37] and Sarcoma 180 [38]. Their specific activities of membrane-bound ^{125}I were increased 5–6-fold over that of the cell homogentaes. In contrast, the ^{125}I specific activity of L-1210 plasma membrane obtained by our method is increased 12–14-fold as shown in Table I and the enzyme assay data in Table II show a 20-fold increase in 5′-nucleotidase and alkaline phosphatase activities. These data indicate that L-1210 plasma membranes isolated by the present method are at least 2–3 times purer than those obtained by using the two-phase polymer system.

The plasma membranes prepared by our method have a predominant peptide with a molecular weight of 50 000 when analyzed on sodium dodecyl sulfate gel electrophoresis. The predominance of this peptide was not observed in the membrane preparation obtained using the two-phase polymer system [34]. This may be due to the difference in the purity of membranes prepared by these two methods because following our isolation procedure we have found that the peptide with molecular weight of 50 000 showed a definite increase in proportion to the purity of the membrane fractions (Fig. 8).

We have shown the discrete ^{125}I pattern of surface membrane proteins of HeLa cells [9, 10] using 7.5 % cylinder gel at neutral pH. However, a broad radioac-

tive pattern of L-1210 cells was obtained with a peak at molecular weight 80 000 using the acrylamide gel system shown in Fig. 5B. The resolution of the radioactive pattern of L-1210 cells was improved by changing the gel composition and the conditions of electrophoresis. Slab gel electrophoresis has been shown to have great sensitivity in resolving microsomal cytochrome P-450 subunits [19]. Using the slab gel and a discontinuous buffer system a discrete radioactive labeling pattern as well as a discrete protein pattern was obtained for the L-1210 membranes. The results show that the choice of a gel electrophoresis system for analysis is very critical.

Because of the differences in the gel electrophoresis system and in the membrane preparations used for glycoprotein analysis, the pattern of the periodic acid-Schiff stain of our plasma membranes is somewhat different from that of the membrane glycoprotein extract of Hourani et al. [34]. The three weakly stained and closely located glycoprotein bands with molecular weight around 90 000 observed in our 7.5 % slab gel seem to correspond to the major band at 84 000 in their 5 % cylinder gel system. The other three minor bands reported in their paper were not observed in our experiments.

The isolation method presented in this paper provides higly purified plasma membrane fractions of L-1210 cells for the analysis of membrane protein components and for the characterization of the exposed surface membrane proteins. The isolation of the plasma membrane of P-388, another murine leukemia cell line, has also been worked out using ^{125}I labeling and a different homogenization medium. The membrane proteins of these two murine leukemica cell lines, L-1210 and P-388, are compared [39]. We are now scaling up the number of starting cells so that plasma membrane fractions with high purity can be prepared in large quantities.

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