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CELLULAR FRACTIONATION AND ISOLATION OF THE PLASMA MEMBRANE OF BURKITT'S LYMPHOMA CELLS

JERRY D. BOLAND and JOHN TWETO *

Biological Markers Program, National Cancer Institute, Frederick Cancer Research Center, Frederick, MD 21701 (U.S.A.)

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Summary

A procedure for cellular fractionation and preparation of plasma membrane from a Burkitt's lymphoma cell line is described. This procedure involves homogenization with a Polytron in buffered isotonic sucrose, and separation of cellular fractions by differential and isopycnic centrifugation in sucrose. The isolated plasma membrane fraction contains 44% of the cellular cholesterol, 50% of the ouabain-sensitive ($Na^+ + K^+$)-ATPase activity, 43% of the γ-glutamyltranspeptidase activities and 16% of the phospholipid. This fraction contains only 3% of cellular protein and is contaminated with less than 4% of the total cellular activities of microsomal, lysosomal, mitochondrial, Golgi and soluble marker enzymes. The cholesterol : phospholipid molar ratio of the crude plasma membrane is 0.56. The membranes in this fraction are in the form of vesicles. Further purification of plasma membrane is achieved by sucrose density gradient centrifugation and results in a 25- to 30-fold enrichment of plasma membrane markers. Plasma membrane markers band in these gradients between 1.10 and 1.15 g/cm^3.

The distribution patterns in the cell fractions of 18 cellular constituents are quantitatively determined. Most constituents are found to distribute in a fashion consistent with the results obtained in other systems. Thymidine-5'-phosphodiesterase (phosphodiesterase I), esterase, nucleoside diphosphatase

* To whom correspondence should be addressed at (present address): P.O. Box 490, Rapid City, SD 57701, U.S.A.

Abbreviation: Tricine; *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

and glucose-6-phosphatase, however, are shown to be poor markers of membrane fractions in this system.

Lactoperoxidase-catalyzed iodination was used to identify several plasma membrane proteins which are exposed at the surface. After separation of labeled polypeptides by sodium dodecyl sulfate gel electrophoresis, the predominant labeled protein was identified as the heavy chain of IgM. Several lesser labeled proteins were observed.

Introduction

An understanding of the properties of the lymphoma cell surface may be useful in order to gain insight into the function of the lymphocyte in the immune response [1] and to understand the nature of the changes which take place upon malignant transformation. We have employed a Burkitt's lymphoma cell line as a model system of the lymphocyte surface. These cells are related to B-lymphocytes, since they have surface receptors for complement component C3, Epstein-Barr virus and the F_c region of immunoglobulin [2—6]. They also express histocompatibility antigens and immunoglobulin on their surface [2—5]. In order to study the molecular nature of these and other cell surface features, it is necessary to purify the plasma membrane.

Several methods have been developed for the purification of plasma membrane from lymphoid cells [7—13]. These methods differ chiefly in the method of homogenization of the cells. Recently a method has been described for the purification of plasma membrane from the Raji line of Burkitt's lymphoma [13]. This method is novel in that the cells were loaded with glycerol prior to hypotonic lysis. The quality of the membrane prepared by this method is difficult to assess, since the intracellular localization of plasma membrane and endoplasmic reticulum markers is not validated in the Raji cell system. The plasma membrane marker thymidine 5′-phosphodiesterase (phosphodiesterase I, EC 3.1.4.1) and the endoplasmic reticulum markers glucose-6-phosphatase (EC 3.1.3.9) and esterase (EC 3.1.1.2) are each found to be significantly enriched in the soluble cytoplasm [13].

In this report we describe a simple procedure for purification of plasma membrane from another line (CA46) of Burkitt's lymphoma. In order to be certain of the quality and yield of plasma membrane, we have measured the distributions after fractionation of 18 biochemical markers of intracellular compartments. We show that the enzymes, thymidine-5′-phosphodiesterase, glucose-6-phosphatase, esterase and nucleoside diphosphatase (EC 3.6.1.6), are not valid markers of membranes in this cell system. In addition, we identify, by means of lactoperoxidase-catalyzed iodination of intact cells, several plasma membrane polypeptides which are exposed on the cell surface.

Methods

Materials. Substrates and biochemicals for all enzyme assays were obtained from Sigma Chemical Co., St. Louis, MO. Sucrose (RNAase-free) and Tris (free base) were obtained from Schwartz/Mann, Orangeburg, NY. Triton X-100 was

obtained from Serva, Accurate Chemical and Scientific Corp., Hicksville, NY. Diaminobenzoic acid was obtained from Eastman Organic Chemicals, Rochester, NY. Orcinol was obtained from Sigma. Cholesterol, 5-α-cholestane, Chromsorb W AW (80/100 mesh) and Dexsil GC 300 were obtained from Applied Science Laboratories, Inc., State College, PA. *N,O*-bis(trimethylsilyl)-trifluoroacetamide, containing 1% trimethylchlorosilane was obtained from Pierce Chemical Co., Rockford, IL. Radioactive chemicals, [7(n)-^{3}H]cholesterol (9.5 Ci/mmol), [G-^{3}H]ouabain (14.7 Ci/mmol), [5-^{3}H]uridine (20 Ci/mmol), uridine diphospho[U-^{14}C]galactose (322 mCi/mol) and ^{125}I (carrier-free) were obtained from Amersham, Arlington Heights, IL. Other chemicals were obtained from commercial suppliers and were reagent grade. Tissue culture medium and fetal calf serum were obtained from Gibco, Grand Island, NY.

Cell cultures. Burkitt's lymphoma cells (CA46 line) were provided by Dr. Ian Magrath, National Cancer Institute (U.S.A.). They were maintained in stationary suspension culture in RPMI 1640 medium, supplemented with heat-inactivated fetal calf serum (10%) and l-glutamine (1.5 g/l). This cell line contained no Epstein-Barr virus nuclear antigen or detectable Epstein-Barr genome (Magrath, I.T., Pizzo, P.A., Whang-Peng, J., Alabaster, O., Freeman, C.B. and Novikovs, L., unpublished observations) and was negative for mycoplasma. Cultures were maintained at a cell density of $0.3—3 \cdot 10^6$/ml and were discarded after 20 passages. New cultures were initiated from a stock of frozen cells.

Cell fractionation. Early log-phase cultures ($2—3 \cdot 10^9$ cells) were harvested by centrifugation at $250 \times g$ for 10 min. The cell pellet was washed three times in 100-ml ice-cold homogenizing buffer (0.25 M sucrose, 4 mM $MgCl_2$, 10 mM Tris, pH 7.8). All subsequent operations were performed at ice temperature. The pellet from the third wash was resuspended in 50 ml of homogenizing buffer and divided into two equal aliquots in 50-ml conical centrifuge tubes. At this stage there were approx. $2 \cdot 10^9$ cells with a viability of greater than 97% as judged by trypan blue exclusion.

Each aliquot was homogenized for 60—90 s with a Polytron (Brinkman Instruments, Model PCU 2) at a power setting of 8. Cellular breakage was assessed by measuring the lactate dehydrogenase activity of an aliquot of homogenate. Homogenization was continued for 15-s intervals until the lactate dehydrogenase activity of the homogenate was approx. 90% of that measured in the presence of 0.1% Triton X-100. Nuclear breakage was assessed by counting with a phase contrast hemocytometer. At 90% cell breakage 40—60% of the nuclei were intact.

Fractionation. The procedure used for fractionation of the homogenate is described in Fig. 1.

Enzyme assays. All assays were performed at 37°C except as noted. Each activity was measured at two or more time points and two or more protein concentrations to insure linearity of kinetic parameters.

Ouabain-sensitive ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) was assayed as described [14] with modifications [15], using ouabain octahydrate (0.5 mM) to determine the ouabain-sensitive ATPase activity. The reaction was stopped by addition of 0.25 ml of ammonium molybdate (40 mM in 4 N H_2SO_4). Phosphate was determined after measurement of the absorbance at 820 nm by using a standard curve.

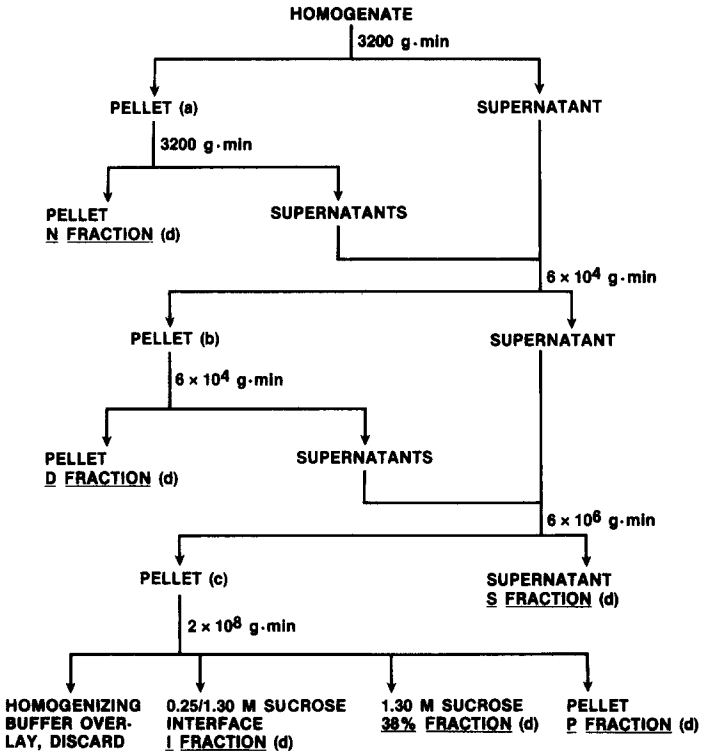

Fig. 1. Schematic representation of the preparation of Burkitt cell fractions. The homogenate was prepared as described in Methods. (a) The pellet from the first centrifugation was twice resuspended in homogenizing buffer (10 ml) by Dounce homogenization (B pestle) and was recentrifuged. The supernatants from this step were combined with that from the first centrifugation. (b) This pellet was resuspended twice in homogenization buffer (10 ml) by Dounce homogenization, recentrifuged, and the supernatants combined with that from the initial 60 000 × g · min centrifugation. (c) This pellet was resuspended in 7 ml of 38% (1.30 M) sucrose (1.17 g/cm^3 in 10 mM Tris, pH 7.8) using a Dounce homogenizer (B pestle). The suspension was overlaid with homogenizing buffer and centrifuged (15 h at 35 000 rev./min) in an SW40 rotor (Beckman Instrument Co.). Following centrifugation the homogenizing buffer overlay was discarded. The material at the 0.25 M/1.30 M interface was removed with a syringe and a 15 gauge needle and diluted with homogenizing buffer. The material in the 1.30 M sucrose and the pellet were also saved. (d) The pellet fractions were resuspended in homogenizing buffer by Dounce homogenization, and the volumes of all fractions were noted. The fractions were divided into aliquots and frozen at —80° C.

Nucleoside diphosphatase (EC 3.6.1.6) was determined [16] by phosphate released, after isobutanol extraction [15].

γ-Glutamyltranspeptidase (EC 2.3.2.1) was assayed [17] by measuring absorbance of the samples at 410 nm. The amount of product liberated was determined using the absorption coefficient of 9.2 $cm^{-1} \cdot mM^{-1}$ [18].

Phosphodiesterase I and II (EC 3.1.4.1), esterase (EC 3.1.1.21), ouabain-sensitive *p*-nitrophenylphosphatase (EC 3.6.1.3), and *N*-acetylglucosaminidase (EC 3.2.1.36) were estimated by measuring at 400 nm the release of *p*-nitrophenol from *p*-nitrophenylthymidine 5′-phosphate, *p*-nitrophenylthymidine 3′-phosphate, *p*-nitrophenylacetate, *p*-nitrophenylphosphate and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, respectively. An absorption coefficient of 17 $cm^{-1} \cdot mM^{-1}$ was assumed [19].

Phosphodiesterase I was assayed in a final volume of 1 ml. The reaction mixture contained 0.1 M glycine-NaOH, pH 8.5, 0.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM *p*-nitrophenylthymidine 5'-phosphate, and enzyme [20].

Phosphodiesterase II was assayed with *p*-nitrophenylthymidine 3'-phosphate (0.5 mM) in a reaction mixture composed of 30 mM sodium citrate, pH 4.5, 0.5 mM $MgCl_2$, 0.1% Triton X-100, and enzyme in a final volume of 1 ml.

Esterase and *N*-acetylglucosaminidase were determined in a volume of 1 ml [16].

The hydrolysis of *p*-nitrophenylphosphate by $(Na^+ + K^+)$-ATPase was measured [21]. Ouabain-sensitive activity was determined by incubation of the enzyme for 90 min in the presence of ouabain (1 mM) prior to the addition of substrate to start the reaction.

Lactate dehydrogenase (EC 1.1.1.27) was assayed at 25°C [22]. The reaction mixture contained 0.9% NaCl and 0.1% Triton X-100. The oxidation of NADH was observed for 5 min at 340 nm. An absorption coefficient for reduced NADH of 6.22 $cm^{-1} \cdot mM^{-1}$ was assumed [23].

Glucose-6-phosphatase (EC 3.1.3.9) was assayed [24]. NaF (0.8 mM) and EDTA (0.8 mM) were included to inhibit nonspecific phosphatase activity [25]. The acid-soluble phosphate was measured as described [26].

Succinate dehydrogenase (EC 1.3.99.1) was assayed at 25°C [27] using phenazine methosulfate and 2,6-dichlorophenolindophenol. The reaction was followed by observing the change in absorbance at 600 nm. The concentration of phenazine methosulfate for maximum velocity was determined for each assay and was approx. 0.3 mg/ml. Triton X-100 (0.05%) was included in each assay. The absorption coefficient for 2,6-dichlorophenolindophenol was 10.5 $cm^{-1} \cdot mM^{-1}$ [27].

NADH cytochrome *c* reductase (EC 1.6.2.1) was assayed at 25°C [16] by observing the change of absorbance at 550 nm. The absorption coefficient for reduced cytochrome *c* was 19.6 $cm^{-1} \cdot mM^{-1}$ [16]. The reaction was not inhibited by the addition of rotenone (0.5 μm) [28].

NADH diaphorase (EC 1.6.99.3) was assayed at 25°C in the same buffer system as for cytochrome *c* reductase except that $K_3Fe(CN)_6$ (0.66 mM) was substituted for cytochrome *c* [23]. The oxidation of NADH was followed at 340 nm.

Galactosyl transferase (EC 2.4.1.22) was assayed by measuring the formation of *N*-[^{14}C]acetyllactosamine from UDP[^{14}C]galactose and *N*-acetylglucosamine [29].

Radioactive labeling. Cells were labeled with [^{3}H]uridine by incubation of cultures (1 l) in medium containing the isotope (0.25 μCi/ml of culture) for 20 h prior to harvest. [^{3}H]Uridine radioactivity was measured after absorption of 50-μl aliquots of the fractions on filter papers and washing with ice-cold 5% trichloroacetic acid. Total incorporation was 5.8 μCi. The identity of the cellular label as RNA was indicated by the complete solubilization of acid-precipitable radioactivity in 0.5 N $HClO_4$ at 75°C for 20 min.

Cells were labeled with [^{3}H]cholesterol by incubation of cultures (1 l) in the presence of the isotope (0.25 μCi/ml of culture) for 16 h before harvest. The radioactivity in 50-μl aliquots of the cell fractions was measured directly after solubilization in Protosol (New England Nuclear). Total incorporation was

0.5 μCi. Incorporated label was identified as cholesterol, after extraction as described below for unlabeled cholesterol, by thin-layer chromatography on silica gel in two solvent systems (petroleum ether/diethyl ether/formic acid, 80 : 20 : 2, v/v/v; and 1% methanol in cyclohexane).

Cells were labeled with [^{3}H]ouabain by incubation of cultures (1500 ml) with the isotope (0.05 μCi/ml of culture) at 37°C for 1 h prior to harvest. Radioactivity of the fractions was measured directly by dissolving 50-μl aliquots in Protosol (New England Nuclear). Total incorporation was 0.1 μCi.

For enzymatic iodination [30] the cells were carefully washed to remove dead cells and debris by the following procedure. Cultures were harvested as described above, and the cell pellet was resuspended in ice-cold Dulbecco's phosphate-buffered saline (35 ml). The resuspended cells were then placed in a 50 ml conical centrifuge tube and underlaid with 10-ml homogenizing buffer and then with 0.5 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The cells were then pelleted at 260 × g for 4 min and the supernatant removed by aspiration. The cells on the Ficoll-Paque cushion were resuspended in phosphate-buffered saline and washed once more. The washed cells were then resuspended at a concentration of $1 \cdot 10^7$/ml in ice-cold phosphate-buffered saline supplemented with Tricine (10 mM), glucose (100 mM), KI (10 nM), lactoperoxidase (Sigma, 10 μg/ml) and $Na^{125}I$ (4 μCi/ml). The reaction mixture was adjusted to pH 8.2 prior to use. The reaction was started by addition of glucose oxidase (Sigma Type II, 5 μg/ml) and was terminated after 30 min by underlaying with homogenizing buffer containing 0.02% NaN_3, and then with Ficoll-Paque, followed by centrifugation at 260 × g for 4 min. The supernatant was discarded, and the cells were washed twice more in homogenizing buffer. Following iodination, cell viability was 98—99% as judged by trypan blue exclusion.

The covalently incorporated label was identified [31] as monoiodotyrosine (85—90%) or as neutral lipid, soluble in $CHCl_3/CH_3OH/H_2O$, 10 : 10 : 3, v/v/v (10—15%). Protein-bound radioactivity was determined after trichloroacetic acid precipitation [31] and extraction with $CHCl_3/CH_3OH/H_2O$.

All ^{3}H and ^{14}C radioactivity determinations were made in duplicate in a liquid scintillation counter using a toluene-based scintillation fluid containing 2,5-diphenyloxazole (4 g/l), p-bis[2-(5-phenyloxazolyl)benzene (0.05 g/l) and 10% ethanol.. Quench of all samples was essentially the same. Counting efficiency for ^{3}H was 57%. Samples were counted for sufficient time to attain an error of less than ±4% at 2σ. ^{125}I was counted in a gamma spectrometer with an efficiency for ^{125}I of 80%. Samples were counted to an error of ±1% at 2σ.

Chemical determinations. Nucleic acids were extracted as described [32]. An aliquot of the extracts was used for the determination of RNA [33] using yeast RNA as a standard. Another aliquot was used for the determination of DNA [32] using calf thymus DNA as a standard.

Lipid was extracted as described [34,35]. Phospholipid phosphorus was determined as described [26,36]. Phospholipid was calculated assuming a phosphate content of 4% and an average phospholipid M_r of 775 [13,37].

Cholesterol in the lipid extract was determined after evaporation of the chloroform phase to dryness under a stream of nitrogen at 37°C. The dried extracts were treated with 50 μl of a mixture of distilled pyridine and N,O-

bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane) (1 : 1, v/v) for 30 min at room temperature and were then analyzed with a gas chromatograph equipped with a flame ionization detector. Separation was achieved with a glass column (3 ft. × 2 mm) packed with 3% Dexsil GC 300 on Chromsorb W AW (80/100 mesh). The injection temperature was 250°C, and the column temperature was 270°C. 5-α-Cholestane was added to the samples as an internal standard. The areas under the cholesterol and cholestane peaks were used as a measure of the amount of material in each peak by comparison with standards. The average retention time of 5-α-cholestane was 2.83 min and of cholesterol, 5.15 min. Mass spectral analysis was used to verify the identity of the cholesterol peak.

Protein was determined [38] using bovine albumin as a standard.

Electron microscopy. Samples of the fractions were prepared for electron microscopy as described [39]. Sections were made in a plane parallel to the axis of centrifugation.

Results

Analysis of the cell fractions

The cell fractions were examined by electron microscopy. The N fraction was composed of nuclei, whole cells, nuclear fragments and debris. The D fraction was composed of some broken nuclei, densely stained chromatin and a large amount of debris. The P fraction contained large numbers of ribosomes, some empty vesicles, some vesicles containing densely stained material, and chromatin. The I fraction (Fig. 2) was composed of empty vesicles, some

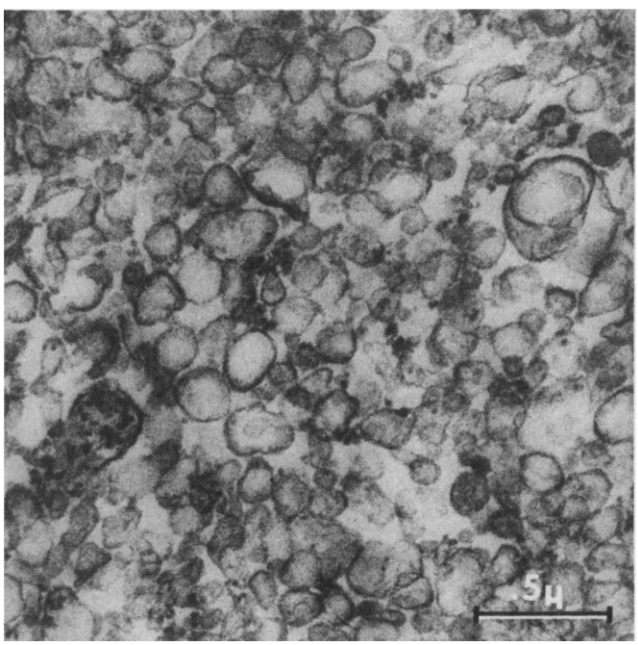

Fig. 2. Electron micrograph of material in cell fraction I (see Fig. 1). Magnification, ×37 300.

TABLE I

DISTRIBUTION OF CONSTITUENTS AFTER FRACTIONATION OF BURKITT'S LYMPHOMA CELLS

Cells fractions were isolated, and the constituents were determined as described in Methods. The results are given as averages of the numbers of experiments listed ± a value indicating the maximum observed deviation from the average. The amount of a constituent in a fraction is expressed as a percentage of the sum of the amounts of that constituent found in the N, D, P, 38%, I and S fractions. The recovery is the same sum, as a percentage of the amount found in the homogenate.

Constituent	Number of experiments	% found in cell fraction:						% recovery
		N	D	P	38%	I	S	
Protein *	6	25.3 ± 8.4	18.6 ± 2.7	14.0 ± 3.6	5.0 ± 0.9	2.8 ± 0.5	34.3 ± 2.3	90.0 ± 10.4
Phospholipid	4	20.6 ± 9.3	23.2 ± 3.8	23.1 ± 2.3	6.0 ± 4.3	16.1 ± 3.3	10.9 ± 3.1	96.4 ± 11.7
DNA	3	64.9 ± 11.3	26.9 ± 10.4	7.6 ± 1.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	74.4 ± 1.8
RNA	3	35.7 ± 10.3	19.0 ± 3.9	25.2 ± 2.3	9.2 ± 7.0	1.5 ± 0.6	9.2 ± 3.8	78.0 ± 6.2
[^{3}H]Uridine	1	28.7	21.1	25.2	13.6	1.7	9.7	89.3
Cholesterol	2	6.1 ± 2.7	10.5 ± 0.5	19.7 ± 3.1	10.4 ± 1.4	44.3 ± 1.6	9.0 ± 3.4	94.2 ± 1.0
[^{3}H]Cholesterol	1	4.6	8.8	24.7	13.0	43.7	5.2	98.2
γ-Glutamyltranspeptidase *	7	6.1 ± 3.3	6.0 ± 1.2	20.2 ± 4.6	14.5 ± 3.3	43.3 ± 5.3	9.8 ± 2.7	108.4 ± 12.0
($Na^+ + K^+$)-ATPase	2	9.2 ± 2.6	1.4 ± 0.1	20.0 ± 3.2	15.3 ± 6.5	50.9 ± 2.9	3.2 ± 3.2	93.0 ± 5.2
p-Nitrophenyl phosphatase	1	10.1	13.1	19.2	12.1	45.5	0.0	93.8
[^{3}H]Ouabain	2	3.8 ± 1.5	3.8 ± 0.5	20.1 ± 2.4	14.0 ± 0.7	50.3 ± 0.8	8.0 ± 0.8	95.0 ± 2.8
NADH Diaphorase	4	22.5 ± 9.9	33.6 ± 1.4	28.6 ± 7.3	4.8 ± 4.5	2.7 ± 1.8	7.7 ± 4.0	96.7 ± 11.0
NADH cytochrome *c* reductase	3	45.8 ± 11.9	28.8 ± 10.3	14.1 ± 4.2	3.6 ± 1.4	3.6 ± 2.0	4.2 ± 1.6	75.5 ± 6.9
Glucose-6-phosphatase	2	40.2 ± 0.7	14.4 ± 0.7	13.3 ± 0.7	2.4 ± 0.3	2.5 ± 0.2	27.3 ± 1.3	78.3 ± 2.8
Succinate dehydrogenase	3	37.6 ± 7.2	30.3 ± 3.2	18.3 ± 4.5	3.4 ± 2.7	0.6 ± 0.3	7.9 ± 4.0	97.4 ± 0.7
N-Acetyl-β-glucosaminidase	3	24.5 ± 2.5	5.7 ± 1.5	7.5 ± 1.3	4.5 ± 0.7	3.6 ± 0.8	54.2 ± 3.4	99.7 ± 13.4
Phosphodiesterase I	3	18.9 ± 4.0	6.7 ± 2.0	5.2 ± 2.4	3.2 ± 1.1	3.5 ± 2.5	61.6 ± 2.8	92.0 ± 5.0
Phosphodiesterase II	2	30.0 ± 1.0	8.4 ± 1.1	9.7 ± 0.8	3.0 ± 0.8	1.8 ± 1.7	46.2 ± 3.4	92.9 ± 8.0
Lactate dehydrogenase	3	3.8 ± 2.2	0.7 ± 0.3	2.0 ± 0.2	1.6 ± 0.3	1.7 ± 0.6	85.4 ± 2.1	94.4 ± 14.6
Nucleoside diphosphatase	1	3.6	1.2	5.4	6.7	11.7	71.4	122.3
Esterase	1	13.4	3.2	3.9	2.2	3.9	73.5	85.4
Galactosyltransferase	2	15.0 ± 6.4	30.8 ± 5.2	25.2 ± 5.4	4.8 ± 2.8	4.2 ± 0.6	20.0 ± 2.8	105.2 ± 5.6

* The results for these constituents are given as the mean ± S.D.

vesicles containing densely stained material and some ribosomes. No other organelles were identifiable either due to low numbers or because they were fragmented by homogenization. The amounts of several enzyme and chemical constituents of the cell fractions are shown in Tables I and II. The distribution patterns of the markers are sufficiently distinct to allow assignments of groupings. Three markers of the plasma membrane, γ-glutamyltranspeptidase [7,40,41], ouabain-sensitive ($Na^+ + K^+$)-ATPase [7,14,40,41] and cholesterol [42—44], are found to be almost entirely in particulate fractions which contain membranes (membrane material is marked by the presence of phospholipid). More than 40% of these markers are found in the I fraction. Phosphodiesterase I activity, which is a plasma membrane marker in other cell systems [40—41,45], does not fractionate like a plasma membrane component in this system.

The relative specific activities of these plasma membrane markers are listed in Table III. The ouabain-sensitive ($Na^+ + K^+$)-ATPase distribution represents an average of the activity measured in three different ways: by ouabain-sensitive hydrolysis of ATP, ouabain-sensitive hydrolysis of *p*-nitrophenylphosphate [7,21] and by the amount of radioactivity found in the fractions after incubation of cells in culture medium containing [^{3}H]ouabain [46]. It can be seen (Table I) that each of these approaches gives similar results when the data are

TABLE II

ABSOLUTE VALUES OF MEASUREMENTS

The amounts of the constituents were determined as described in Methods. The absolute values refer to $1 \cdot 10^9$ cells. They are expressed as the average of the number of experiments ± a value which gives the largest observed deviation from the average and are the sums of the absolute values obtained in the N, D, P, 38%, I and S fractions. The values for protein, DNA, RNA and cholesterol are given as mg. Phospholipid phosphorous is given as μmol. Enzyme units are expressed as μmol product formed/min per mg protein.

Constistuent	EC	Number of experiments	Absolute value
Protein		7	96.4 ± 13.4 *
Phospholipid phosphorous		4	4.6 ± 0.9
DNA		3	4.4 ± 0.7
RNA		3	3.1 ± 0.6
Cholesterol		2	1.10 ± 0.2
γ-Glutamyltranspeptidase	2.3.2.1	7	0.025 ± 0.008 *
($Na^+ + K^+$)-ATPase	3.6.1.3	2	0.27 ± 0.02
p-Nitrophenylphosphatase	3.6.1.3	1	0.04 ±
NADH Diaphorase	1.6.99.3	4	11.8 ± 3.2
NADH cytochrome *c* reductase	1.6.2.1	3	0.46 ± 0.05
Glucose-6-phosphatase	3.1.3.9	2	0.53 ± 0.11
Succinate dehydrogenase	1.3.99.1	3	0.93 ± 0.26
N-Acetyl-β-glucosaminidase	3.2.1.30	3	1.0 ± 0.5
Phosphodiesterase I	3.1.4.1	3	0.08 ± 0.02
Phosphodiesterase II	3.1.4.1	2	0.06 ± 0.01
Lactate dehydrogenase	1.1.1.27	3	177 ± 53
Nucleoside diphosphatase	3.6.1.6	1	0.05
Esterase	3.1.1.2	1	1.30
Galactosyltransferase	2.4.1.22	2	0.49 ± 0.12

* Values refer to the mean ± S.D.

TABLE III

RELATIVE SPECIFIC ACTIVITIES OF PLASMA MEMBRANE MARKERS IN BURKITT'S LYMPHOMA CELL FRACTIONS

Data were derived by expression of the data of Table I as specific activities, i.e., percentage of total activity in a fraction/percentage of total protein in a fraction. The specific activities were multiplied by the value: percentage recovered activity/percentage recovered protein. Homogenate specific activity = 1.0.

Constituent	Relative specific activity in cell fraction					
	N	D	P	38%	I	S
γ-Glutamyltranspeptidase	0.29	0.39	1.7	3.5	18	0.35
Cholesterol *	0.23	0.56	1.6	2.4	17	0.24
$(Na^+ + K^+)$-ATPase **	0.29	0.26	1.5	2.9	18	0.14
Phosphodiesterase I	0.76	0.36	0.37	0.65	1.3	1.8

* Represents an average of the two methods in Table I.
** Represents an average of the three methods in Table I.

converted to relative values. Similarly, the cholesterol distribution is an average of the results of two approaches: direct measurement by gas-liquid chromatography and observation of the distribution of radioactivity after incubation of cells in culture medium containing [^{3}H]cholesterol. The data of Table I show that these approaches give similar results.

γ-Glutamyltranspeptidase, $(Na^+ + K^+)$-ATPase and cholesterol fractionate similarly and are enriched in the I fraction 17- to 18-fold compared to homogenate (Table III). The endoplasmic reticulum markers [7,23,28,42—45], NADH diaphorase, NADH cytochrome *c* reductase and RNA, are enriched relative to the homogenate in the particulate fractions (Table IV). Phosphodiesterase I (Table III), esterase, nucleoside diphosphatase, and glucose-6-phosphatase (Table IV) do not show enrichment patterns characteristic of membrane-bound enzymes.

The enrichment of authentic endoplasmic reticulum markers in the I fraction (Table IV) indicates some contamination with internal membranes. Lysosomal markers, *N*-acetyl-β-glucosaminidase [7,42] and phosphodiesterase II

TABLE IV

RELATIVE SPECIFIC ACTIVITIES OF ENDOPLASMIC RETICULUM MARKERS IN BURKITT'S LYMPHOMA CELL FRACTIONS

Data were derived from Table I as described in the legend to Table III.

Constituent	Relative specific activity in cell fraction					
	N	D	P	38%	I	S
NADH Diaphorase	0.95	1.9	2.2	1.0	1.0	0.24
RNA *	1.2	0.95	1.6	1.8	0.52	0.25
NADH cytochrome *c* reductase	1.5	1.3	0.85	0.61	1.2	0.10
Glucose-6-phosphatase	1.4	0.67	0.83	0.42	0.78	0.69
Esterase	0.50	0.16	0.26	0.42	1.3	2.0
Nucleoside diphosphatase	0.18	0.09	0.52	1.8	5.7	2.8

* Represents an average of the two methods in Table I.

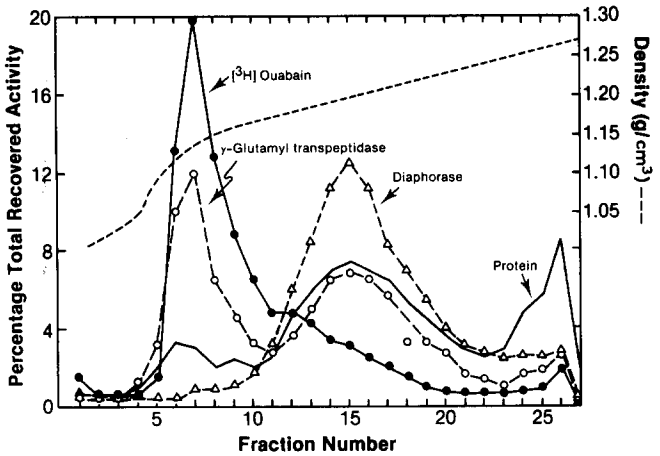

Fig. 3. Analysis of the material in the P fraction by isopycnic centrifugation in a sucrose density gradient ($Na^+ + K^+$)-ATPase was measured by labeling Burkitt's lymphoma cells with [^{3}H]ouabain as described in Methods. Material in the P fraction was suspended in homogenizing buffer and layered on a sucrose gradient (35—55%, w/w, in 10 mM Tris, pH 7.8). Sample was centrifuged in an SW40 rotor (Beckman Instrument Co.) at 40 000 rev./min for 15 h (10^{12} rad^2/s). Following centrifugation, 0.5 ml fractions were removed, starting from the top. The constituents were determined as described in Methods. Sucrose density was measured on every third fraction by refractometry and is indicated by the dotted line. The solid line represents protein, 7.8 mg applied, 91% recovered; △- - - - -△, diaphorase, 3.1 μmol/min applied, 100% recovered; ○- - - - -○, γ-glutamyltranspeptidase, 7 nmol/min applied, 62% recovered; ○———○, [^{3}H]ouabain, 6000 cpm applied, 135% recovered.

[45], are largely absent from this fraction. Contamination by mitochondrial marker, succinate dehydrogenase [27], DNA and soluble marker lactate dehydrogenase is very low. A significant amount of the Golgi marker [29], galactosyl transferase, is found in the I fraction. Golgi contamination may explain the enrichment for nucleoside diphosphatase in the I fraction (Table III) [47].

Subfractionation of the I and P fractions

The I and P fractions were further analyzed on sucrose density gradients. Most of the [^{3}H]ouabain (Fig. 3) bands at a density below 1.17 g/cm^3, where only a small amount of protein and very little diaphorase are found. Somewhat less than half of γ-glutamyltranspeptidase activity (Fig. 3) is found at a density lower than 1.17 g/cm^3, while the rest of the activity is found with most of the protein and diaphorase activity at higher densities. The diaphorase activity (Fig. 4) is found largely at a density higher than 1.15 g/cm^3, and the lysosomal marker, *N*-acetylglucosaminidase, is found (Fig. 5) mostly below a density of 1.09 g/cm^3.

Iodination

Burkitt's lymphoma cells were labeled by iodination. The surface specificity can be seen from the data in Table V. The distribution of protein-bound label is similar to that of γ-glutamyltranspeptidase. The difference in the percentage of label found in the ND fraction and the I fraction when radioactivity is compared with enzyme marker is due to the incorporation of label into extracel-

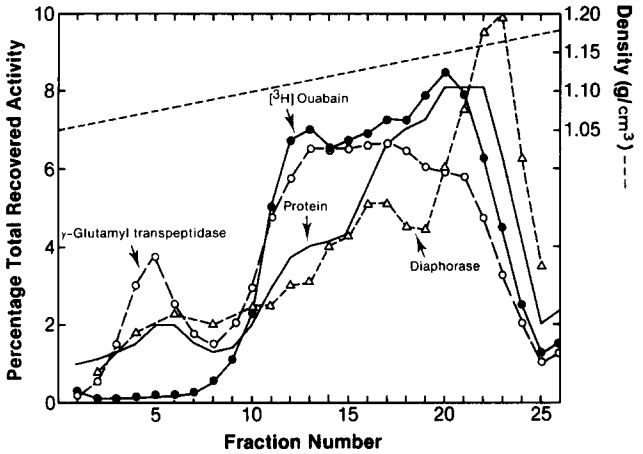

Fig. 4. Analysis of the material in the I fraction by isopycnic centrifugation in a sucrose density gradient. Procedures were as described in the legend to Fig. 3 except the I fraction was analyzed on a 15—40% sucrose gradient. Fractions were 0.5 ml. ------, sucrose density; ———, protein, 3.4 mg applied, 76% recovered; △- - - - -△, diaphorase, 0.8 μmol/min applied, 80% recovered; ○- - - - -○, γ-glutamyltranspeptidase, 20 nmol/min applied, 97% recovered; ○———○, [^{3}H]ouabain, 47 000 cpm applied, 89% recovered.

lular debris which is not removed by cell washing. The labeled extracellular debris sediments at 60 000 × g · min, and is composed of material which migrates at or near the tracking dye when analyzed by sodium dodecyl polyacrylamide gel electrophoresis (data not shown). Extraction of the cell fractions with Triton X-100 (1%) solubilizes 83% or more of the covalently bound ^{125}I from all but the ND fraction where only 54% is extracted. The Triton-insoluble label from

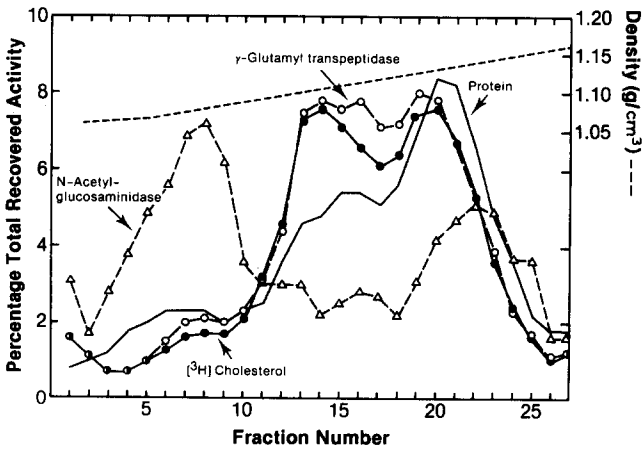

Fig. 5. Analysis of the material in the I fraction by isopycnic centrifugation in a sucrose density gradient. Procedures were as described in the legends to Figs. 3 and 4 except that the I fraction material was obtained from Burkitt's lymphoma cells which had been labeled with [^{3}H]cholesterol as described in Methods. ------, sucrose density; ———, protein, 2.2 mg applied, 90% recovered; ○- - - - -○, γ-glutamyltranspeptidase, 17 nmol/min applied, 85% recovered; ○———○, [^{3}H]cholesterol, 106 000 cpm applied, 93% recovered; △- - - - -△, *N*-acetylglucosaminidase, 200 nmol/min applied, 125% recovered.

TABLE V

DISTRIBUTION ANALYSIS OF ^{125}I IN BURKITT'S LYMPHOMA CELL FRACTIONS AFTER LACTOPEROXIDASE-CATALYZED IODINATION

Burkitt's lymphoma cells were labeled with ^{125}I as described in Methods. Labeled cell fractions were prepared as described in Methods, except that the preparation of a separate N fraction was omitted. The material in the N fraction was sedimented with D fraction (60 000 × g · min) and the combined fractions are referred to as ND fraction. For preparation of the Triton X-100 extracts, aliquots of the fractions were mixed with detergent (final concentration 1%) and centrifuged at 165 000 × g for 45 min. The resulting supernatant (Triton X-100 extract) was saved. Antiserum against Burkitt plasma membrane was prepared in a goat by immunization with material in the I fraction. Immune precipitable radioactivity in the Triton X-100 extracts of each fraction was determined by addition of a previously determined excess of either the IgG fraction of goat anti-I fraction or goat anti-IgM (Miles/Yeda). Quantitative precipitation was effected by addition of an optimal amount of anti-goat IgG prepared in a donkey (Miles/Yeda). Nonspecific precipitation was measured by substituting goat preimmune IgG or serum for either anti-I fraction or anti-IgM antibodies in parallel experiments. After washing, the radioactivity in the immune precipitates was measured, and specific precipitation was determined by subtraction of nonspecific background.

Constituent	Total from homogenate (cpm × 10^{-6})	% recovered activity in fraction				
		ND	P	38%	I	S
γ-Glutamyltranspeptidase	—	21	17	8.9	46	6.5
Total protein-bound ^{125}I	18.2	27	16	11	40	5.9
Triton X-100-extractable ^{125}I	15.5	18	16	15	44	6.6
Anti-I fraction-precipitable ^{125}I	8.13	16	17	15	47	4.6
Anti-IgM-precipitable ^{125}I	1.91	11	14	14	59	1.7

the ND fraction migrates with the tracking dye. Triton X-100-solubilized radioactivity distributes in the same fashion as γ-glutamyltranspeptidase, indicating that this label is localized in the plasma membrane.

Antibody prepared by immunization of a goat with the material from the I fraction can be used to selectively precipitate Burkitt cell proteins which are exposed on the cell surface (Table V). The material which is recognized by this antibody can be seen in Fig. 6, lane D. Most, but not all, of the ^{125}I-labeled proteins are removed from a Triton X-100 extract of I fraction (compare lanes B, C and D) after passage over a Sepharose 4B column to which anti-I fraction IgG has been covalently bound. Of the several labeled proteins which are specifically bound by the anti-I fraction antibody, one predominates. This protein migrates at the position of the heavy chain of IgM (lane E) at an apparent M_r of 77 000 and can be specifically precipitated with anti-IgM antiserum (data not shown). From Table V, it can be seen that iodinatable IgM is localized in the plasma membrane and comprises at least 12% of the covalently bound ^{125}I in the Triton X-100 extracts.

Other major labeled proteins are seen migrating at apparent M_r values of 42 000, 33 000, 27 000 and less than 12 000 (Fig. 6). The M_r 25 000 band is a doublet, probably the Ig light chain and a component of the HLA-linked antigens [50]. The three other major labeled species may be other components of the HLA system [50].

Discussion

The procedure described in Methods for the preparation of plasma membrane from Burkitt's lymphoma cells is simple, reproducible and results in a

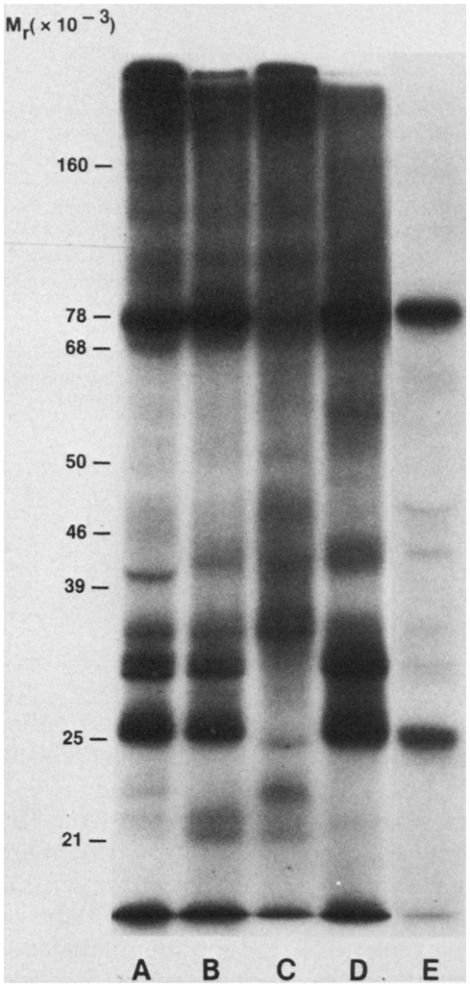

Fig. 6. Autoradiogram of iodinated proteins after sodium dodecyl sulfate polyacrylamide gel electrophoresis of Burkitt's lymphoma plasma membrane proteins. Material from the I fraction of the experiment described in Table V is analyzed in lane A. This I fraction was extracted with Triton X-100 and the soluble material (lane B) was passed over a column of Sepharose 4B to which anti-plasma membrane IgG has been covalently bound. Material which was not bound to the affinity column was eluted with phosphate-buffered saline containing 1% Triton X-100 (lane C). Bound material was eluted with the same buffer, but containing 3 M NaSCN (lane D). Recovery of label from the column was 80%. Lane E is the pattern obtained with iodinated [48] pentameric IgM (Cappel Laboratories) (separate exposure). The migration positions of molecular weight standards are indicated: RNA polymerase (M_r 165 000; M_r 39 000), conalbumin (M_r 78 000), serum albumin (M_r 68 000), IgG (heavy chain M_r 50 000, light chain M_r 25 000), actin (M_r 46 000), soybean trypsin inhibitor (M_r 21 500). Analysis was on a 9% acrylamide gel using the system of Laemmli [49].

fraction (I) containing about 3% of the cellular protein, 20% of phospholipid and 45% of the plasma membrane markers. Contamination of this fraction with other cellular components is low. The major contaminating components of the I fraction, lysosomal and microsomal material, can be largely removed, and the plasma membrane markers significantly enriched by isopycnic centrifugation in a sucrose gradient (Figs. 4 and 5). Plasma membrane markers found at a density

of 1.11 g/cm^3 in such a gradient are enriched 25- to 30-fold in specific activity over the values in the homogenate. This enrichment is similar to that found for plasma membrane markers in other lymphoid cell plasma membrane preparations [1,7—13,44], and indicates relatively pure plasma membrane. The purified plasma membrane of the I fraction contains 183 μg cholesterol/mg protein. This is similar to the ratios obtained in other lymphoid cell plasma membrane preparations which range from 160 to 460 μg/mg [1,8,12,13,44]. The value of this ratio depends upon the extent of purification of the membrane. The more highly purified membranes obtained from the gradients in Fig. 4 have a calculated ratio of 274 μg/mg which is somewhat higher than the value of 194 μg/mg found in RAJI cells, and is in good agreement with the value (263 μg/mg) reported for human lymphocytes [12].

The phospholipid : protein ratio in the I fraction is intermediate to that reported by most other workers [8—11,13,44] who have found ratios varying between 330 and 2790 μg/mg. We find 660 μg/mg. Our cholesterol and phospholipid values were not obtained on the same preparations. However, assuming that they are representative, they correspond to a molar ratio of cholesterol-to-phospholipid of 0.20 in the homogenate and 0.56 in the I fraction. These values are lower than the values reported for a homogenate of the P3J line of Burkitt's lymphoma cells (0.30) [37] and the values of 0.97 and 0.75 reported for the purified membrane of RAJI cells [13] and human lymphocytes [12], respectively. Our cholesterol : phospholipid ratio is within the range (0.34—1.03) reported by others [8—13,44] for lymphoid cells from several species.

Homogenization results in significant organelle breakage since 50—60% of the lysosomal enzyme activities is found in the soluble fraction, and since 35% of the cellular DNA fails to sediment in the nuclear fraction. Mitochondrial breakage may be the explanation for the succinate dehydrogenase activity found in the high-speed pellet and in the soluble fraction. It can be argued that homogenization which gives rise to organelle breakage might also detach loosely bound enzymes from cellular membranes and might thus be the explanation for the soluble activities of phosphodiesterase I, esterase, nucleoside diphosphatase and glucose-6-phosphatase. We have tested this possibility by homogenization of cells in hypotonic buffer using a Dounce homogenizer and fractionation by a scheme similar to that described in Methods. Both homogenization techniques give rise to similar particulate and soluble distribution patterns of these enzymes and of NADH diaphorase (data not shown). Variation in the homogenization procedure does not significantly affect the yield or enrichment of plasma membrane markers in the I fraction. Such variation does affect the distribution patterns of other membrane markers. Prolonged homogenization results in increased fragmentation of components with a shift in the distribution of some constituents toward the fractions obtained at higher centrifugal fields, and gives rise to significant variability in distribution from preparation to preparation.

It was necessary to determine the distributions of a large number of constituents because of the inconsistencies in the distributions of putative plasma membrane and endoplasmic reticulum markers. The results in Tables II and III clearly indicate that phosphodiesterase I, esterase and nucleoside diphosphatase

are not useful membrane markers in our system. Glucose-6-phosphatase also is of limited utility due to significant soluble activity. These results conflict with those obtained using another line (RAJI) of Burkitt's lymphoma [13]. In that report, 17% of the phosphodiesterase I activity is found in the cytosol compared to 60% found here. Similarly, 65% of esterase activity and 14% of glucose-6-phosphatase activity are found in the soluble cytosol compared to the values of 85% and 27%, respectively, reported here. These differences may be a characteristic of different cell lines.

Approx. 10% of the total cellular γ-glutamyltranspeptidase activity, however, appears to be associated with the microsomes (Fig. 3 and Table I; the P fraction contains only 20% of the total activity of this enzyme), and approx. 1% of the total activity may be associated with the lysosomes (Table I, Figs. 4 and 5). Ouabain-sensitive ($Na^+ + K^+$)-ATPase appears better to reflect the distribution of plasma membrane (Table I, Fig. 4). We have shown that the difficult assay for this enzyme can be bypassed by following the distribution of [^{3}H]ouabain, which has been bound to the cells prior to homogenization. This technique offers a simple means of determining ($Na^+ + K^+$)-ATPase in routine analysis. Similarly, the use of metabolically incorporated [^{3}H]cholesterol allows the rapid determination of the distribution patterns of cholesterol (Table I, Fig. 5).

Radioactivity incorporated into intact cells by lactoperoxidase-catalyzed iodination is not a specific marker for the plasma membrane in our system. This is due to the presence of extracellular debris from the growth medium which cannot be wholly removed by washing. More extensive washing than we have employed here compounds the problem of specificity because of increased cell death. Labeled debris can be separated from plasma membrane label due to its insolubility in 1% Triton X-100 (Table V). Protein-bound ^{125}I found in the Triton X-100 extracts of the labelled cells does represent label specifically incorporated into membrane proteins (Table V). The lack of lactoperoxidase probe specificity seen here points out the necessity of demonstrating plasma membrane localization of iodine label when lactoperoxidase-catalyzed iodination is used with a new cell system.

Finally, we have prepared antibody against purified Burkitt cell plasma membrane. This antibody is cytotoxic, in the presence of complement, toward Burkitt's lymphoma cells and another lymphoid cell line, NC-37, but not toward non-lymphoid human cell lines (data not shown). This antibody specifically binds to several of the iodinatable cell surface proteins of Burkitt cells (Fig. 6) and is presently being used to identify and purify Burkitt's lymphoma-specific cell surface proteins.

Acknowledgments

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