A method has been developed for the rapid large scale isolation of plasma membranes and intact nuclei from RAJI lymphoid cells utilizing hypotonic lysis of cells after intracellular loading with glycerol followed by combined flotation-sedimentation within a discontinuous sucrose gradient. Nuclei may be isolated in about 1 h and plasma membranes in about 6 h from 1 to 20 g of cells. Intact nuclei, obtained in 90 to 95% yield based on lysed cells, was isolated by differential centrifugation and contained 16% DNA and about 30% of total cell sialic acid.

The less dense plasma membrane fraction was isolated by centrifugation onto a cushion of 38% sucrose (d = 1.1683) and subsequently resolved into two subfractions. The less dense vesicles had an average d = 1.127 and showed a 5-fold increase in specific activity for thymidine phosphodiesterase while the more dense d = 1.151 had a 20-fold concentration of enzyme activity. Activity of enzymes indicative of contamination with lysosomes, microsomes, mitochondria, and cytoplasm was negligible in these plasma membrane fractions. The less dense vesicles had a cholesterol:phospholipid ratio of 0.97 which was higher than that of the more dense vesicles (0.69). Otherwise, the analytical values for the two types of membrane vesicles were similar as both fractions contained 16% DNA and about 30% of total cell sialic acid.

Twenty-five per cent of the total cell sialic acid was in the plasma membrane fractions.

The initial event in lectin-induced transformation of lymphocytes is the interaction of mitogen with the cell surface. In the first paper of this series (1) we have shown that one of the effects of transformation of peripheral blood lymphocytes with phytohemagglutinin (PHA) of Phaseolus vulgaris is a 20- to 40-fold stimulation of incorporation of radioactive D-glucosamine and that three different size classes of radioactive product were directly associated with the lymphoid cell membrane but that the third component, although transiently associated with the plasma membrane, was secreted from the cellular cytoplasm (3). These studies have required the rapid and frequent isolation of plasma membranes on a large scale. While a multitude of methods for the isolation of plasma membranes has been developed in the past few years these are frequently not applicable to cell types other than that for which they were developed or require inordinately long times to process small quantities of material which are insufficient for chemical characterization.

The method reported here permits the rapid processing of from 1 to 20 g of cells. The disruption procedure is based on the glycerol-lysis method initially developed for human platelets (4), but has been extensively revised to reflect the greater permeability of lymphoid cells to glycerol. A novel method has been developed for fractionation of the subcellular organelles which involves the simultaneous flotation and sedimentation in a stepwise sucrose gradient to effect a rapid purification of plasma membrane vesicles. In addition, this method permits the isolation of intact cell nuclei in high yields from the original homogenate.

MATERIALS AND METHODS

Cell Cultures

RAJI, a human Burkitt lymphoma cell line, was obtained from American Type Culture Collection (ATCC) cell repository and had undergone 100 subcultures. This suspension cell line was described by ATCC as being virus-free as determined by electron microscopy and free of Epstein-Barr virus as determined by immunofluorescent staining. The methods for cell culture were as previously described (3). Not more than 30 subcultures were carried out; after this the lines were discontinued and new cultures established from the original stock.

The cells were harvested under aseptic conditions in the log phase of growth (1.5 × 10⁶ cells/ml). They were gently centrifuged from the culture medium in a Sorvall RC 3 centrifuge which had been warmed to 30°C at 2000 rpm (800 × g, for 10 min, HB-4-rotor). The cells were washed twice at 37°C with a volume of Earle’s balanced salt solution equal to 10% of the original culture volume.

The concentrations of all sucrose solutions were determined from their refractive indices and adjusted as required since the percentage of water in various batches of ribonuclease-free sucrose (Schwartz and Mann) was quite variable. A refractometer (Bausch and Lomb) with a 20°C constant temperature bath was used. A concentrated

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1 Observation to be published elsewhere.
Differential Centrifugation

adjusted to give the appropriate sucrose concentration, corresponding to 60 to 64% sucrose solution. This solution was then adjusted to give the appropriate sucrose concentration, as calculated from refractive index values, in a final concentration of 10 mM Tris buffer (pH 7.4).

Lysis Method

The washed cells were resuspended in Earle's balanced salt solution equivalent to 1% of the original culture volume. A solution of glycerol (90% w/v in Earle's balanced salt solution) was added in three equal increments 5 min apart to give a final concentration of 30% glycerol. All of the above operations were carried out at 30-37°C. Five minutes after the last addition of glycerol, the cell suspension was cooled in an ice bath for 2 min and from this point on all operations were carried out at 4°C. The glycerol-loaded cells were centrifuged from the suspension in 250-ml plastic bottles using a Sorvall RC-2B centrifuge at 8000 rpm (2700 x g) for 10 min and the supernatant solution was decanted and discarded.

A volume of buffer (10 mM Tris/HCl, pH 7.4, containing 1.0 mM MgCl₂, 1.0 mM CaCl₂) equal to 0.7 to 1% of the original culture volume was rapidly added to the pelleted cells with vigorous agitation and the mixture was allowed to stand in an ice bath for 5 min.

Fractionation Procedure

The fractionation scheme is summarized in Fig. 1.

Differential Centrifugation

In order to remove the nuclei the cell lysate was centrifuged at 700 x g in the Sorvall RC-2B centrifuge, HS-4 rotor at 2000 rpm for 10 min. The supernatant solution was removed by aspiration. The pelleted nuclei were resuspended in the same volume of the lysis buffer and centrifuged as before.

The supernatant solutions (“nuclei-free lysate”) combined after pelleting and washing the nuclei were centrifuged at 3300 x g in the Sorvall RC-2D, HPR-4 rotor for 10 min at 4000 rpm in order to remove a debris fraction comprising mitochondria. The supernatant solution (“membrane lysate”) was carefully removed by aspiration prior to further purification of the membrane fraction.

Ultracentrifugation

Step 1 – The crude membrane fraction was obtained by sedimentation onto a sucrose cushion: 33 ml of supernatant solution (membrane lysate from Part A, Fig. 1) was placed in cellulose nitrate ultracentrifuge tubes (1/2 x 3 1/2 inches) and 38% sucrose (5 ml) was layered underneath the lysate using a Pasteur pipette. The sample was centrifuged in the Sorvall OTD-2 ultracentrifuge using a Beckman SW 27 swing-out rotor for 1 1/2 h at 26,500 rpm (90,000 x g). A band, designated as crude membrane fraction, formed at the interface. The supernatant solution above this band was removed completely as possible by aspiration and the crude membrane fraction and sucrose cushion were decanted. A residual pellet, which remained adherent to the bottom of the tube, termed “debris,” was resuspended for assay.

Step 2 – Further purification involved the simultaneous flotation and sedimentation of membrane fractions within a discontinuous sucrose gradient and their separation from contaminating subcellular organelles.

The crude membrane fraction was adjusted to a refractive index of n = 1.3829 to 1.3847 (corresponding to 31 to 32% sucrose concentration) by adding the calculated volume of concentrated (approximately n = 1.451) sucrose solution. The following solutions, buffered with 10 mM Tris/HCl, pH 7.4, were pipetted into a 38-ml centrifuge tube (2.6 x 9 cm) in the following order: (a) 7 ml of 38% sucrose solution; (b) 20 ml of crude membrane suspension; (c) 15% sucrose solution was then added until the tube was balanced (about 12 ml). The sample was then centrifuged for 2 h in the Sorvall OTD-2 ultracentrifuge using a fixed angle rotor (T-965) at 83,000 rpm (430,000 x g).

Wash

The membranes were washed, as described in the legend of Fig. 1, to remove residual contaminating material which, in the case of the floated vesicles was lysosomal particles, and with the sedimeted vesicles was lysosomal and microsomal particles. This procedure has been used by other investigators to remove such contaminating structures.

Fractionation

Fractionation was effected by gently lowering thin (1-mm diameter) polyethylene tubing to the bottom of the centrifuge tube and pumping out the tube from the bottom by a Sigmamotor pump at maximum speed (100 ml/min): the fractions were monitored by use of an LKB Uvicord fraction collector. Refractive index (0.8 ml) were collected in 2-ml conical cups (Technicon, Tarrytown, N.Y.) using an LKB Ultrorace fraction collector. Refractive indices were used to correlate sucrose density with the 980 nm absorbance pattern. The fractions were stored at -20°C until use.

Enzyme Assays

The enzyme assays were performed at 37°C using the methods outlined below. In each case, the reaction was shown to be linear with time and proportional to the amount of enzyme preparation used. Calculations are based on international units of activity in which 1 unit of enzyme yields 1 μmol of product/h.

Lactic Acid Dehydrogenase (EC 1.1.27) – This enzyme was used as a cytoplasmic marker (6). The activity was measured by following the decrease in 340 nm absorbance due to the conversion of NADH to NAD, a parallel reaction in the lactic acid dehydrogenase conversion of pyruvate to lactate (7).

Succinate Dehydrogenase (EC 1.3.99.1) – This activity has been shown to be typical of mitochondria (8). Succinate dehydrogenase activity was determined using 2,6-dichloroindophenol as the electron acceptor (9). The initial change in absorbance was used to calculate units of activity.

Phosphodiesterase (Nonspecific) (EC 3.1.4.18) – This enzyme was used for screening samples. It is a plasma membrane marker (10) and was assayed by the method of Koerner and Sinsheimer (11).

Thymidine-5’-phosphodiesterase (EC 3.1.3.35) – This enzyme was used as a marker for the plasma membrane (11). The substrate used was thymine 5-p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) and the change of absorbance at 400 nm was observed at 30, 60, and 90 min, basically following the procedure of Erecinska et al. (11).

Glucose-6-phosphatase (EC 3.1.3.9) – Glucose-6-phosphatase was used as a microsomal marker (12) and was measured by the procedure of Nordlie and Arion (13) by following the release of inorganic phosphate by the method of Bartlett as modified by Ames (14).

β-N-Acetylglucosaminidase (EC 3.2.1.30) – This enzyme was used as a lysosomal marker (15) and was measured by the increase of absorbance at 420 nm at 20, 40, and 60 min using p-nitrophenyl glucosaminide (Sigma) as a substrate (16).

Esterase – This enzyme phosphonomonoesterase (EC 3.1.3.2) was used as a marker of microsomal material (17). The method used for assay was that described by Earl and Korner (9).

Chemical Analyses

The samples were pooled and dialyzed exhaustively against distilled water. Each was lyophilized, then dried in vacuo at 100°C over P₂O₅, for 18 h, cooled to 25°C, and weighed.

Protein

Protein was determined for enzymatic studies by the procedure of Lowry et al. (18) using bovine serum albumin as a standard and by amino acid analysis for chemical studies, using norleucine as an internal standard.

Amino Acids

Amino acid analyses were performed after hydrolysis in constant boiling HCl at 108°C under nitrogen for 24 h at a weight concentration of 1 μg of sample/ml. The sample was evaporated to dryness and dissolved in 0.2 N citrate buffer, pH 2.2. The amino acid analyses were performed using a JEOL model JLC-6AH amino acid analyzer.

Carbohydrate

Sialic acid was determined by the thiobarbituric acid assay (19) after hydrolysis in 0.1 N H₂SO₄ at 80°C for 1 h. In order to assay sialic acid in the presence of large amounts of DNA, a new method was designed using radioactive sialic acid as an internal standard and preferentially extracting the chromophore attributable to DNA.

2 Total hexose was determined by the phenol/sulfuric acid method.

M. Jett, manuscript in preparation.
of Dubois et al. (20) using equimolar amounts of galactose and mannose as a standard. Hexosamines were determined by the Elson-Morgan procedure modified by Boas (21) after hydrolysis in 4 N HCl for 2 h in sealed tubes.

**Lipids**

Total lipids were determined gravimetrically after extraction three times with chloroform/methanol (2:1 v/v) at room temperature for 18 h and partitioning against an equal volume of distilled water by the method of Folch et al. (22) and colorimetrically by the sulfophosphovanillin reaction (23). Total phosphorus and organic-extractable phosphorus were determined after perchloric acid combustion and the Schmidt-Morgan procedure modified by Boas (21) after hydrolysis in 4 N HCl at 110°C for 18 h and partitioning against an equal volume of distilled water.

**Cholesterol** was determined by the Lieberman-Burk reaction as modified by Watson (26).

**Nucleic Acids**

DNA was determined by measuring fluorescence (Turner I fluorometer) with ethidium bromide (27). Incubation at 37°C for 1 h in 0.3 N KOH was used to eliminate fluorescence attributable to RNA. The fluorescence after hydrolysis represented the DNA in the sample. Calf thymus DNA solutions, made fresh daily, were used for a standard. The difference before and after hydrolysis was a measure of the RNA in the sample. RNA was also determined by the Schmidt-Thannhauser method as described by Munro and Fleck (28). For this assay, type III RNA (Sigma) was used as a standard since it was equivalent to the average of solutions of AMP and UMP.

**Electron Microscopy**

Fractions were routinely fixed with 1.25% glutaraldehyde in 4% sucrose, 0.05 M phosphate buffer (pH 7.3) for 18 to 20 h. After fixation, the samples were washed in buffer (0.05 M phosphate buffer, pH 7.3) for about 30 min and then postfixed in buffered 1% osmium tetroxide. Samples were dehydrated in ascending concentrations of ethanol and finally in propylene oxide and embedded in Epon 812 (29, 30). Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined in a Hitachi HU-12 transmission electron microscope.

**RESULTS**

**Lysis and Fractionation** — The procedure described under "Materials and Methods," and summarized in Fig. 1, afforded a rapid isolation of a fraction containing intact cell nuclei and the separation of two populations of plasma membrane vesicles. The first step, differential centrifugation, was effective in removing nuclei and cell debris. Then a crude membrane fraction was obtained by centrifugation onto a 38% sucrose pad (d 1.1683). The crude membrane fraction was adjusted with concentrated sucrose to achieve a density intermediate between that of the two bands (31 to 32% sucrose solution; d 1.1339 to 1.1386) and this fraction was introduced in a centrifuge tube so as to be intermediate between sucrose solutions of 15% concentration (d 1.0610) and 38% concentration (d 1.1683). This simultaneous sedimentation-flotation step was devised to separate two populations of plasma membrane vesicles according to their different buoyant densities. The lighter of the two bands floated to the 32 to 15% interface while the heavier sedimented onto the cushion of 38% sucrose and lysosomal contaminants floated to the top of the tube or were held in the 32% phase.

**Fig. 2** shows the profile of a typical experiment. Phosphodiesterase (nonspecific) activity was associated with the sedimented and the floated material with very little being retained in the middle fraction. The protein profile shows three major fractions, the sedimentsed and floated vesicles, and the intermediate fraction. This intermediate fraction consists of contaminating organelles, primarily lysosomal material as determined by β-N-acetylglucosaminidase activity (15) which reached a peak at an elution volume of 18 ml. The minor protein peak at 38 ml also had a large amount of β-N-acetylglucosaminidase activity associated with it.

The major contaminants left after the sedimentation-flotation gradient were lysosomal (β-N-acetylglucosaminidase) with both bands and microsomal (esterase) with the sedimented band. In order to see if the contaminating activities were intimately associated with the plasma membranes, the two fractions were each subjected, separately, to isopycnic density centrifugation on 20 to 45% sucrose gradients. Fig. 3A shows that a small amount of β-N-acetylglucosaminidase activity was coincidental with the peak of phosphodiesterase activity of the floated vesicles. Fig. 3B shows a minor peak of β-N-acetylglucosaminidase activity to coincide with the phosphodiesterase activity of the sedimented vesicles. A characteristic pattern of the specific activities of the two enzymes for the sedimented band is shown in Fig. 3C. The specific activity of β-N-acetylglucosaminidase associated with the plasma membrane fraction is slightly less than that in whole cells while...
brane fractions and to see the distribution, compared to the N-acetylglucosaminidase activity, and 20% of the eraser fractions, respectively, but a high percentage of the total cells are selected out by this procedure. This may be due to the fact that the uptake covers an additional 4 to 6% of the cells, their lysis properties cells in this fraction. Although increased centrifugation re-

mately 12 to 15% of all the enzymatic activities are found in whole cell homogenate, of a variety of marker enzymes (listed about 20% of the N-acetylglucosaminidase activity in the procedure. Indeed, 95% of the phosphodiesterase activity but only 92% of the phosphodiesterase (PDE) activity, and esterase activities (Table II). Lactic acid dehydrogenase, esterase, and β-N-acetylglucosaminidase were concentrated only in the cytosol fraction.

The chemical composition of certain fractions is shown in Table III. The two plasma membrane fractions contained 2.88% of the whole cell protein. The sedimented membrane fraction is the larger of the two, comprising nearly two-thirds of the total protein and of the dry weight. In both plasma membrane bands, the protein/lipid ratio in near unity while the cholesterol/phospholipid ratio approaches unity for the floated band but is 0.69 for the sedimented band. A ratio of 0.7 was found for lymphocyte plasma membranes from tonsil tissue (31) although most reported values approach unity (5, 32). The highest cholesterol content in the floated band may account for its higher buoyant density (33). Only minute amounts of DNA and RNA, 0.06 and 0.5%, respectively, of the total cell content occur in the plasma membrane fractions. The sialic acid content of the combined plasma membrane fractions is 20% of the total cell sialic acid (Table III) and there is little difference in the overall carbohydrate content of the two bands (Table IV).

Nuclear Fraction - The recovery of intact nuclei, as determined in 10 preparations by light and phase contrast microscopy, was 75 to 80%. Since there is a loss of 15% of the cells in the glycerol phase (Table I), the yield of intact nuclei is 90 to 95% based on lysed cells.

The nuclear fraction contains 63% protein and 16% DNA (Table IV) and represents 92% of the total DNA of the intact cell when corrected for cell loss in the glycerol phase (Table III).

Electron Microscopy - Electron micrographs of various fractions are shown in Fig. 4. The nuclei (Fig. 4a) are somewhat oblong and are 4 to 6 μm in width and approximately 9 μm in length. For the most part, the double nuclear membrane is intact.

The "membrane lysate" (see Fig. 1A) preparation shows an abundance of plasma membrane vesicles (Fig. 4b) plus various ribosomal and lysosomal contaminants.

The sedimented plasma membrane vesicles (Fig. 4c) have an average diameter of 3 to 4 μm. Some ribosomes appear to be an integral part of the plasma membrane while a few other particles seem to be trapped inside the vesicles. Fig. 4d shows the same sample after the final washing by dilution to 4% sucrose concentration and subsequent pelleting (Fig. 1, Step D).

The floated plasma membrane vesicles (Fig. 4e) are approximately 2 μm in diameter. There are a few granular particles scattered throughout the fraction.

**DISCUSSION**

Since the study of membrane-related phenomena requires the isolation of plasma membranes, a variety of techniques have been developed for cell disruption and subcellular frac-
Lymphoid cells have been disrupted by most of the conventional techniques including nitrogen cavitation, Potter-Elvehjem and Dounce homogenization with and without chemical stabilization, mechanical shear devices, and chemical-destabilizing agents; the merits of these various procedures have been reviewed (5, 32, 33).

Optimal cell disruption requires that a balance be achieved between cell breakage and preservation of intracellular organelles (primarily nuclei). In addition the effect on biologic functions such as antigenic and enzymatic activities must be considered. The method we describe results in 85% cell lysis. This degree of cell disruption has been obtained for suspension lymphoid cells using mechanical shear (32, 34) and for solid tissue disrupted by a perforated disc (36) but this latter technique resulted in only 50% lysis (5) when applied to suspension cells.

In evaluating a variety of buffers for lysis of the pelleted, glycerol-loaded cells, 1.0 mM MgCl₂ and 1.0 mM CaCl₂ were used to preserve nuclear integrity. Buffers containing sodium chloride caused membrane clumping either after lysis or upon the addition of sucrose and the percentage of unlysed cells was somewhat greater by both microscopic examination and enzymatic assay when using hypotonic sucrose as compared to 10 mM Tris/HCl buffer.

Several important points have emerged from these studies in order to obtain optimum yields. Cell damage during the washing procedure was decreased by retaining normal culture conditions including maintenance of the temperature at 30–37°C.
(36) and use of balanced salt solutions until the final lysis step.

Upon addition of glycerol to a final concentration of 10%, the
cells immediately began to shrink, as monitored by light and
phase microscopy, and their surfaces became slightly irregu-
lar. By 30 s, 80% of the cells had shrunk, but by 60 s only about
20% were still shrunken and by 90 s, nearly 99% of the cells
had regained their original volume and shape. The second
increment of glycerol, which brought the concentration to 20%,
caus ed similar effects but the changes in size and shape were
less marked. The last addition to 30% produced only slight
changes in cell size and shape. The degree of lysis, based on
enzyme assays and microscopic examination, was not im-
proved by further increase in glycerol concentrations up to
45%.

The outstanding feature of this method is that the nuclei are
recovered intact and quantitatively. Other methods report

### Table I

**Distribution of enzymatic activities in subcellular fractions**

| Enzyme                        | Whole cells (total activity) | Per cent distribution of enzymatic activity in various fractions | Plasma membranes (combined) | Cytosol
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyceral phase</td>
<td>Nucl</td>
<td>Debris</td>
<td></td>
</tr>
<tr>
<td>Tymidine-5'-phosphodiesterase</td>
<td>22</td>
<td>43</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>β-N-Acetylglucosaminidase</td>
<td>67</td>
<td>12</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>90</td>
<td>15</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>Esterase</td>
<td>59</td>
<td>16</td>
<td>39</td>
<td>7</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>585</td>
<td>14</td>
<td>31</td>
<td>36</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>45</td>
<td>9</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*a Total activity is expressed as micromoles of product liberated per h per 9.5 x 10⁶ cells.

*Glyceral phase* is the supernatant solution resulting when the cells are centrifuged after the addition of glycerol.

*Debris* includes the combined pellets from Steps A, B, and C in Fig. 1.

*These figures represent the sum of the activities of the two plasma membrane fractions.

The cytosol fraction represents the material above the crude membrane (Fig. 1B), the material above the floated band and be-
tween the bands (Fig. 1C) and the supernatant solutions of the wash of the membrane vesicles (Fig. 1D).

Our preliminary data, as well as that of others (33), indi-
cated that rapid separation of organelles from the cell lysate
was necessary to increase recovery and decrease contamina-
tion. For this reason, the use of isopycnic centrifugation was
not acceptable for routine work due to the time involved and
the limitations on amounts of starting material which could be
used. We sought a procedure which would rapidly eliminate
most contaminating structures from the plasma membrane
preparation and settle on a preliminary sedimentation onto a
sucrose cushion. Further purification of this crude membrane
fraction was achieved by simultaneous flotation and sedi-
mentation of the membrane within a carefully designed disconti-
nuous sucrose gradient system. First, the buoyant densities of
the individual membrane bands were determined by centrifug-
ation within a continuous linear sucrose gradient. This
showed that the two bands had densities of 1.151 and 1.127.
The crude membrane fraction was then brought to a density
intermediate between that of the two bands by adding a con-
centrated sucrose solution and it was introduced between su-
crose solutions of higher and lower density as described under
"Materials and Methods" and in Fig. 1. On centrifugation, a
residual debris fraction sedimented to the bottom of the tube
while one population of membrane vesicles sedimented to the
interface with the 30% sucrose cushion, and the other floated
to the interface with the 15% sucrose solution; some lysosomal
material floated to the top of the tube.

This method provides a rapid and effective way of separat-
ing and purifying two populations of membrane vesicles. It
should be readily applicable to other cell types after modifica-
tion of the stepwise gradients to reflect the densities deter-
rmined for the individual membrane fractions in pilot exper-
iments.

### Table II

**Comparison of specific activities of various marker enzymes in subcellular fractions**

<table>
<thead>
<tr>
<th></th>
<th>Whole cells (specific activity)</th>
<th>Glyceral</th>
<th>Nuclei</th>
<th>Debris</th>
<th>Plasma membrane</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine-5'-phosphodiesterase</td>
<td>0.04</td>
<td>0.82</td>
<td>0.96</td>
<td>1.2</td>
<td>20</td>
<td>6.7</td>
</tr>
<tr>
<td>β-N-Acetylglucosaminidase</td>
<td>0.12</td>
<td>0.69</td>
<td>0.27</td>
<td>0.3</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>0.18</td>
<td>0.77</td>
<td>0.81</td>
<td>1.5</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Esterase</td>
<td>0.11</td>
<td>0.91</td>
<td>0.87</td>
<td>1.2</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>1.4</td>
<td>0.79</td>
<td>0.69</td>
<td>5.7</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>0.08</td>
<td>0.51</td>
<td>0.03</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*a Specific activity is expressed as micromoles of product per h per mg of protein.

*b For description of fractions, see Table I.

*c Sed, sedimented.
Nuclei and Plasma Membrane Isolation and Characterization

Table III

Chemical composition of whole cells and subcellular fractions: total milligrams and per cent.

Figures in parentheses represent the percentage of that component in the fraction compared to the whole cell homogenate. The 15% loss of cells in the glycerol phase is not taken into account.

<table>
<thead>
<tr>
<th></th>
<th>Whole cell homogenate</th>
<th>Nuclei</th>
<th>Sedimented plasma membrane</th>
<th>Floated plasma membrane</th>
<th>Sum of plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>3406</td>
<td>1463</td>
<td>67.5</td>
<td>30.4</td>
<td>97.9</td>
</tr>
<tr>
<td>Lipidb</td>
<td>656</td>
<td>222</td>
<td>59.7</td>
<td>29.9</td>
<td>89.6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>108</td>
<td>36.5</td>
<td>9.2</td>
<td>15.1</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>599</td>
<td>148</td>
<td>2.6</td>
<td>0.3</td>
<td>2.9</td>
</tr>
<tr>
<td>DNA</td>
<td>496</td>
<td>372</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Neutral hexoses</td>
<td>245</td>
<td>72.9</td>
<td>21.7</td>
<td>13.6</td>
<td>35.3</td>
</tr>
<tr>
<td>Hexosamines</td>
<td>30.4</td>
<td>7.3</td>
<td>2.9</td>
<td>1.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>7.8</td>
<td>2.5</td>
<td>1.2</td>
<td>0.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Sum (mg)</td>
<td>5548</td>
<td>2324.9</td>
<td>165</td>
<td>82.8</td>
<td>247.8</td>
</tr>
<tr>
<td>Total dry weight (mg)</td>
<td>6613.3</td>
<td>2327.49</td>
<td>201.15</td>
<td>97.47</td>
<td>298.61</td>
</tr>
<tr>
<td>% accounted</td>
<td>84</td>
<td>99.9</td>
<td>82</td>
<td>85</td>
<td>83</td>
</tr>
<tr>
<td>% whole cell dry weight</td>
<td>35</td>
<td>3</td>
<td>1.5</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Cholesterol/phospholipid</td>
<td>0.69</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These figures represent the composition of 7.2 x 10⁶ cells.

b "Lipid" represents total neutral and phospholipids minus cholesterol.

Phospholipid was calculated assuming 25 μg of phospholipid/μg of lipid phosphorus (25).

Table IV

Chemical distribution in nuclei and plasma membrane fractions

<table>
<thead>
<tr>
<th></th>
<th>Plasma membrane</th>
<th>Sedimented vesicles</th>
<th>Floated vesicles</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/mg protein</td>
<td>% dry wt</td>
<td>μg/mg protein</td>
<td>% dry wt</td>
</tr>
<tr>
<td>Protein</td>
<td>33.6</td>
<td>31.2</td>
<td>31.2</td>
<td>62.9</td>
</tr>
<tr>
<td>Lipid</td>
<td>894</td>
<td>24.1</td>
<td>982</td>
<td>30.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>135.5</td>
<td>4.6</td>
<td>193.8</td>
<td>6.1</td>
</tr>
<tr>
<td>RNA</td>
<td>38.6</td>
<td>1.3</td>
<td>9.5</td>
<td>0.3</td>
</tr>
<tr>
<td>DNA</td>
<td>3.4</td>
<td>0.1</td>
<td>2.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Neutral hexoses</td>
<td>321</td>
<td>10.8</td>
<td>447</td>
<td>14</td>
</tr>
<tr>
<td>Hexosamines</td>
<td>43.1</td>
<td>1.5</td>
<td>61.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>18.4</td>
<td>0.6</td>
<td>23.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Per cent accounted for</td>
<td>82.2</td>
<td>85.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Since the nuclear membranes contain large amounts of typical plasma membrane enzyme activities, and these membranes vesiculate as do plasma membranes, they are virtually impossible to distinguish or separate from plasma membranes. In fact, it is commonly believed that there is continuity between the plasma membrane and the nuclear membrane (40). For these reasons, a method of cell disruption which leaves intact nuclei is important for any claim regarding localization of components in either nuclei or plasma membrane. The glycerol procedure offers an ideal system for accomplishing this objective. Although the plasma membrane is permeable to glycerol, the nuclear membrane does not appear to be under these conditions. When the whole cells are exposed to a hypotonic buffer following glycerol-loading, the difference in osmotic pressure between the inside and outside of the plasma membrane is so great that the membrane is stripped off and forms vesicles up to about one-fourth the size of the intact cell. The nuclei are recovered in nearly quantitative yield.

The chemical analyses previously reported for lymphoid cell plasma membranes is somewhat fragmentary and incomplete. Warley and Cook (41) using mouse leukemia cells, found 51.8% protein and 48.1% lipid, while Crumpton and Snary (5) report 42% protein, 51% lipid, and 5.5% neutral sugars and sialic acid for the BRI-8 lymphoid cell line. It may be noted that the total analyses approached 100% for the components assayed in these two reports even though certain components were not determined. The dry weight composition of the two plasma membrane fractions isolated in this study are nearly identical with regard to lipid (~30%) and protein (~32%) while the carbohydrate composition (neutral hexoses, hexosamine, and sialic acid) represents 13% of the dry weight for the sedimented and 17% for the floated vesicles. Another difference between the sedimented and floated vesicles is in the
FIG. 4. a, nuclei × 10,000; b, membrane lysate (see Fig. 1B) × 8,000; c, sedimented vesicles × 10,000; d, washed sedimented vesicles × 18,000; e, floated vesicles × 14,500.
RNA composition which is 9 μg/mg of protein in the case of the floated vesicles and 38 μg/mg for the sedimented vesicles ("Sum of the Plasma Membrane," Table III, shows a total of 29 μg of RNA/mg of membrane protein) other investigators have reported 15 to 34 μg of RNA/mg of protein (5, 31, 34, 37) for lymphoid cells.

Small quantities of DNA (0.06% of the whole cell DNA were found in the plasma membrane vesicles of RAJI cells with the DNA/protein ratio being nearly the same (~3 μg/mg of protein) for both the floated and sedimented vesicles. These values are slightly lower than those reported for plasma membranes from other lymphoid cells (6 to 10 μg of DNA/mg of protein; 16, 31, 34, 37). In fact, there has always been some question regarding the presence of DNA in plasma membrane preparations, but Lerner et al. (42, 43) concluded that some DNA was truly a component of the plasma membranes of WIL 2 cultured cells.

Thymidine-5'-phosphodiesterase, thought to be a marker for plasma membranes (11), is enriched in the membrane fractions isolated by this technique, but is not found there exclusively, being present also in isolated nuclei (Table I). ATPase and 5'-nucleotidase which are also thought to be plasma membrane markers have been reported in nuclear fractions by others (41, 44, 45). In addition, 10 to 15% of these enzymes are contaminating the plasma membrane fraction. Recovery of enzymatic data, probably indicate that nuclear membranes dislodged from the membrane and solubilized during cell disruption, or to enzyme which is not sediment, or to enzyme which is dislodged from the membrane and solubilized during cell disruption, it probably represents a truly cytoplasmic form of the enzyme (5, 11). This means that greater than 50% of the "plasma membrane marker" is associated with other fractions. Therefore, recoveries of greater than 50%, based on whole cell enzymatic data, probably indicate that nuclear membranes are contaminating the plasma membrane fraction. Recovery of plasma membranes may be more meaningful if based on a nuclei-free lysate minus the cytosolic form of the enzyme.

RAJI cell nuclei, on the other hand, contain relatively large amounts (39% of total cell activity) of the microsomal marker enzymes, glucose-6-phosphatase and esterase (Table I) which have also been reported as markers of rat liver nuclei (46) and calf liver nuclei (47), respectively. Thirty per cent of the succinate dehydrogenase activity (Table I) is associated with the nuclei. None of these activities are present in significant amounts in the plasma membrane fraction. Further fractionation of the nuclei, with separation of the inner and outer membranes from the intranuclear material, may define the points of similarity and difference to the plasma membrane.

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