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THE ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANES FROM NORMAL AND LEUKAEMIC CELLS OF MICE

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SUMMARY

A rapid method for the isolation of plasma membranes from normal lymphoid and acute lymphoblastic leukaemic cells of AKR mice was developed. The procedure entailed lysis in a hypotonic medium containing borate and EDTA from which a fraction enriched in surface membrane ghosts was obtained by centrifugation. Further purification was achieved by passage of the fraction through a column of glass beads. Electron microscopic examination of thin sections of the isolated material showed that the final fractions contained sheets of membrane devoid of contamination with cytoplasmic organelles. Appropriate marker enzyme activities and chemical assays were used to monitor the procedure. In both types of cell the specific activity of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was increased compared to a cell lysate, but the recovery in this fraction was low. Membranes derived from normal and leukaemic cells were devoid of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) activity as a marker for mitochondria and possessed low amounts (leukaemic membranes 44.4–149.6 nmoles·mg protein^{-1}·min^{-1}; non-leukaemic membranes 10.4 nmoles·mg protein^{-1}·min^{-1}) of NADH oxidoreductase (EC 1.6.4.3) activity as a measure of contamination by endoplasmic reticulum. In preparations from the leukaemic cells, the molar ratio of cholesterol:phospholipid was 0.99.

INTRODUCTION

Several workers[1,2] have demonstrated changes occurring in the glycoproteins of the cell membrane on malignant transformation. From electrokinetic data on normal and leukaemic cells of AKR mice[3] it would appear that there are structural differences in the glycoproteins of these two cell types. In order to be able to study the glycoproteins of these membranes further, as well as to elucidate any other differences in the chemistry of these cell surfaces, it is essential to be able to prepare purified plasma membrane fractions from both the leukaemic and non-leukaemic cells.

A number of methods are now available for the isolation of plasma membranes, some of which entail reducing the membranes to vesicles which may be fractionated by differential centrifugation[4]. Alternatively the plasma membranes may be isolated in the form of sheets generally when natural stabilizing structures are present, as in

liver[5,6], or by the use of chemical stabilization[7]. Recently McCollester[8] has developed a method for the isolation of surface membrane ghosts of Meth. A tumour cells by the preferential breakdown of the cytoskeleton by inhibition of the FAD-dependent enzymes which are considered to stabilize this structure. McCollester[8] describes his membrane fraction in terms of morphological and chemical studies and (using this preparation) was able to demonstrate immunisation of BALB/c mice against tumour specific transplantation antigens. As a control he performed experiments with a plasma membrane fraction derived from leukaemic cells of AKR mice. Although details as to the preparation of the membrane fraction were presented, together with a phase contrast picture of the treated cells, no chemical or enzymatic data were given. In view of the rapidity of the method the technique was re-examined with a view to its use in comparative studies of normal and leukaemic cells in this laboratory. The technique of McCollester[8] was followed carefully but the membrane enriched material obtained still showed a high degree of contamination with cytoplasmic organelles. We therefore adapted the method to include fractionation on a column of glass beads, following which membrane fractions free of contaminating material were obtained. This procedure, which is applicable to both normal and leukaemic cells, is described here. In addition our modification of the McCollester[8] procedure as applied to normal lymphocytes is compared to other methods[9,10] specifically designed for isolating the plasma membrane of this cell type in vesicular form.

MATERIALS AND METHODS

Chemicals

All solutions were prepared in water which had been distilled once in a metal still followed by two distillations in glassware: this water was used within 24 h of the final distillation. Chemicals and materials for enzyme assays were of analytical grade unless otherwise stated and were obtained from British Drug Houses Ltd, Poole, Dorset. NADH, Trizma buffers, ouabain, sodium succinate and reagents for the determination of inorganic phosphorus were purchased from Sigma Chemical Company Ltd, London. Ethacrynic acid was a gift of Merck Sharp and Dohme Ltd, Hoddesdon, Herts. Bovine serum albumin prepared as aprotein standard was obtained from Armour Pharmaceutical Company Ltd, Eastbourne, Sussex. Ficoll (Lot No. 5990) was the product of Pharmacia, Uppsala, Sweden. The latter material was made into a 20% (w/w) aqueous solution and dialysed exhaustively against de- ionised water, then concentrated in a rotary evaporator to give a stock solution of density about 1.2 $g \cdot cm^{-3}$ conductivity ($2.4 \cdot 10^{-5}\ \Omega^{-1} \cdot cm^{-1}$). A sample of glass beads (Superbrite 150) was kindly provided by the 3M Company, London and subsequently a further quantity was purchased from the same supplier.

Preparation of cell suspensions

For the preparation of plasma membranes the following stock solutions were made up: 1 M NaCl, 250 mM $MgCl_2$, 250 mM $CaCl_2$, 10 mM EDTA (pH 9.6) and 500 mM boric acid. 500 mM sodium borate, pH 9.6, was prepared from the stock boric acid solution by using NaOH pellets.

Acute lymphoblastic leukaemic cells were grown as a subcutaneous nodule in AKR mice as described previously[3]. Material was routinely harvested on the fourteenth

day after transplantation and a cell suspension prepared by pressing the tissue gently through a stainless steel gauze (mesh 0.8 mm) with a smooth glass pestle using a solution of 150 mM NaCl containing 50 mM borate, 1.0 mM $MgCl_2$ and 1.0 mM $CaCl_2$, the pH of which was adjusted to 7.2 with 1 M NaOH before making up to the final volume. This solution which was obtained by dilution of stock solutions of the reagents will be referred to as harvesting solution[8]. The resulting cell suspension was collected at the temperature of melting ice. Routinely 15 g (wet wt) of leukaemic tissue obtained from 40 mice were used for each experiment yielding 20 ml of a suspension containing $1 \cdot 10^8$ cells/ml, with a viability in excess of 95% as assessed by the exclusion of erythrosin B. However 64.8 g (wet wt) of material from 100 mice was necessary for preparations required for chemical analysis.

For the preparation of normal lymphoid cells a substrain[3] of AKR mice, resistant to the lymphoblastic leukaemia were used. Routinely 1.2–1.5 g (wet wt) of mesenteric lymph nodes were removed from 40 animals. Swollen or discoloured lymph nodes were not used, the finding of such nodes was a rare occurrence. Suspensions of normal lymphoid cells were prepared by a similar method to that used for the leukaemic tissue. Stainless steel of a finer mesh (0.5 mm) was used. Usually a 10-ml suspension containing $1.1 \cdot 10^8$ cells/ml was obtained.

Smears of these preparations were stained with May–Grunwald Giemsa stain so that contamination with macrophages and granulocytes could be assessed, examination of the slides showed that 99.9% of the cells present were lymphoid in type.

Preparation of plasma membranes

The isolation procedure followed is shown schematically in Fig. 1; it was based on the method described by McCollester[8] for leukaemic cells in which cells are extracted at room temperature, all other steps were carried out at 4 °C. The same method was also used by us for normal lymphoid cells. Cells were removed from suspension by centrifugation at $500 \times g$ for 5 min. The cells were then washed twice in 10 volumes of harvesting solution at $500 \times g$ for 5 min. After the second wash the cells were resuspended in approximately twice their packed cell volume of harvesting solution rather than in extraction solution as recommended by McCollester[8]. This modification was adopted to overcome the loss of material due to cell lysis occurring before the cells were transferred to the final volumes of extraction solution. The resuspended cells were transferred to Erlenmeyer flasks containing a solution of 20 mM borate and 0.2 mM EDTA, pH 9.2, (extraction solution) at room temperature, and stirred gently using a magnetic stirring device. The ratio of packed cells to extraction solution never exceeded 1:200. The stirring was continued for 10 min, then 8 ml of 500 mM sodium borate, pH 9.6, was added to each flask containing 200 ml of extraction solution. The stirring was stopped after a further 5 s and the contents of the flask were quickly filtered through nylon gauze 0.8 mm mesh (obtainable from Henry Simon Ltd, Stockport) into a bottle and centrifuged at $2000 \times g$ for 1.5 h.

After centrifugation the supernatant was discarded and 25 ml of a membrane wash solution, prepared directly from the stock solutions to give a final concentration of 20 mM borate and 1 mM EDTA, pH 9.2, were added to each bottle and the material was resuspended by vigorous shaking. This membrane enriched material

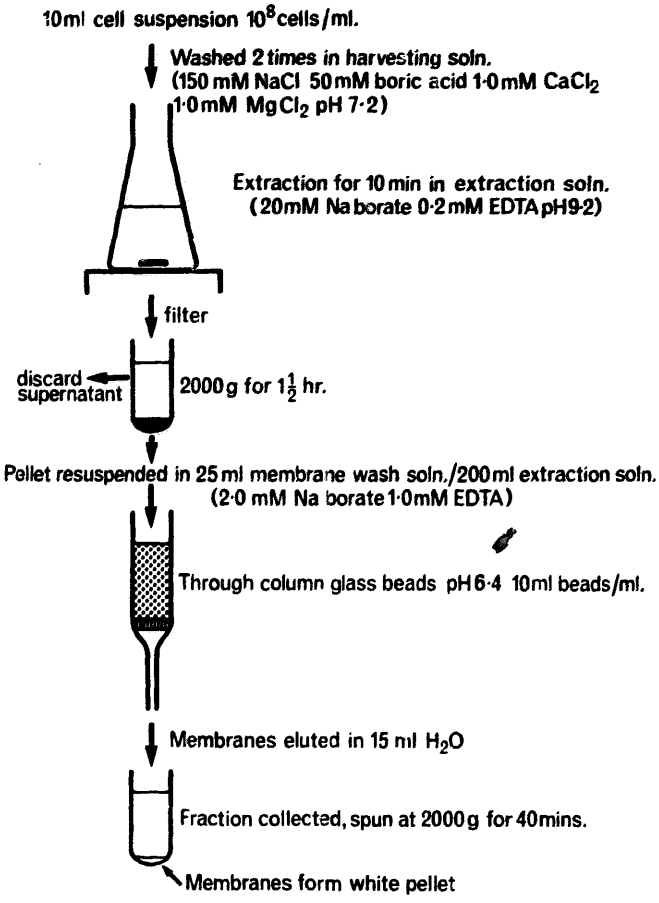

Fig. 1. Flow sheet for the preparation of plasma membranes as modified from the procedure of McCollester[8]. *g* values are as calculated for the centre of centrifuge tube. Details for the preparation of the solutions and the glass beads are given in the text.

was then applied to a column of internal diameter of 2 cm containing glass beads (Superbrite 150). The beads had been previously prepared by soaking for 1 h in 0.1 M HCl before being transferred to the column where they were washed with distilled water until the pH of the eluate was 6.4. The volume of glass beads used was equal to ten times the initial packed cell volume. Following addition of the membrane enriched material to the column, elution was commenced with the application of distilled water. During elution of the column the packing was stirred gently with a fine plastic spatula, care being taken not to disturb the lowest 5 mm of beads. It was found that the membranes eluted within the first 15 volumes of water followed by the elution of other cell debris. The membranes were then pelleted from this fraction by centrifugation at 2000 × *g* for 40 min.

Isolation of plasma membrane fragments from microsomes

Standard washed normal and leukaemic cells[3] were homogenized by nitrogen cavitation as described by Wallach and Kamat[4] using a 'bomb' of the type described by Hunter and Commerford[11]. For normal lymphoid cells the working pressure was reduced from 850 to 750 lb/inch^2 as suggested by Ferber *et al.*[10]. Alternatively in some experiments the normal lymphoid cells were broken in a French pressure cell (Aminco) at a pressure of 5000 lb/inch^2 applied by means

of a hydraulic press. A 'nuclear mitochondrial' pellet was prepared by centrifuging the homogenate at 16000 × g_{av} for 15 min. The resulting supernatant fluid was then carefully removed and centrifuged at 105000 × g_{av} for 45 min to yield a microsomal pellet and a 'cell sap' fraction.

For isolation of fragments of plasma membrane the microsomal pellet was separated on a Ficoll barrier (density 1.082–1.093 g·cm^{-3} at 25 °C) containing Mg^{2+} into a surface membrane fraction and an endoplasmic reticulum fraction, following the method of Wallach and Kamat[4].

The Ficoll barriers were prepared by diluting gravimetrically, a stock solution of Ficoll (density approx. 1.2 g·cm^{-3}). Care was taken to mix the stock solution vigorously with diluent and to remove the air bubbles by centrifugation (500 × g for 5 min) before use. More recently the use of a barrier of dextran-150 (density 1.088 g·cm^{-3} measured at 25 °C) as described by Ferber *et al.*[10] has been used to fractionate microsomal pellets obtained from normal lymphoid cells.

Electron microscopy

Plasma membrane fractions were centrifuged at 2000 × g for 40 min and the pellets fixed in 2.5% glutaraldehyde in 0.09 M sodium cacodylate buffer, pH 7.2, containing 3 mM $CaCl_2$, for 30 min to 1 h at room temperature. The pellets were then washed in buffer, separated into a number of fragments, post-fixed in 1% OsO_4 in veronal–acetate buffer, pH 7.2, for 1 h, stained with 0.5% uranyl acetate in veronal–acetate buffer, pH 5.0, for 1 h, dehydrated in ethanol and embedded in Araldite. Thin sections were stained with lead citrate and examined in a GEC-AEI EM6B electron microscope. Care was taken to ensure that all regions of each pellet were studied.

Assay of enzymic activities

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was measured by the method of Persijn *et al.*[12]. Care was taken to exclude non-specific alkaline phosphohydrolase by the use of phenyl disodium orthophosphate as a selective inhibitor of the latter activity. NADH oxidoreductase (EC 1.6.4.3) was measured by the method of Wallach and Kamat[4] and succinate dehydrogenase (EC 1.3.99.1) activity by the method described by Green *et al.*[13]. Glucose-6-phosphatase was examined by the method of Hubscher and West[14].

Chemical analysis

For the estimation of protein, total lipid, phospholipid and cholesterol, a sample of plasma membrane was dried to constant weight *in vacuo* over P_2O_5 at 20 °C. This material was then extracted twice over a 24-h period with chloroform–methanol (2:1, v/v). The extract was evaporated under a stream of nitrogen and the residue was then dried to constant weight *in vacuo* over P_2O_5 and paraffin wax at 20 °C. The weight of this residue was taken to be the weight of total lipid. The residue was again taken up in chloroform–methanol, and divided, the solvent was evaporated from each portion, one portion was taken up in acetic acid for the measurement of cholesterol by the method of Watson[15], the other portion was incubated with perchloric acid for 1 h at 200 °C and then the liberated phosphorus was determined by the method of Fiske and SubbaRow[16].

Phospholipid was calculated assuming 25 μg phospholipid/μg P_i (ref. 17). The protein content of the fraction which remained after lipid extraction was measured by the method of Lowry *et al.*[18] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Isolation of plasma membranes

In the description of his method for isolating cell surface membranes from Meth A tumour cells and leukaemic cells of AKR mice, McCollester[8] makes the point that there can be no question that the isolation method permits the recovery of surface membranes. McCollester[8] supports this contention by reference to the fact that all the steps in the extraction process can be followed microscopically and that the ghost, consisting of the cell membrane left after the expulsion of the cellular contents, in the initial extraction procedure, is the predominant structure recovered. In addition the chemical analysis published on material from Meth A tumour cells is consistent with what is presently known of the analysis of plasma membrane fractions.

The cell types investigated in the present study responded in an identical manner to the extraction medium to those described by McCollester[8]. Phase contrast microscopy showed that both the normal and leukaemic cells rapidly expelled their cellular contents on treatment with the extraction medium leaving empty ghosts which could be recovered as a surface membrane rich fraction by differential centrifugation (as described by McCollester[8]).

Fig. 2. Typical field of a thin section of material present in the plasma membrane fraction prepared from murine leukaemic cells by the method of McCollester[8]. Magnification × 25000. Electron dense bodies can be seen in addition to sheets of plasma membrane.

Electron micrographs of thin sections of a plasma membrane pellet from leukaemic lymphoblasts produced by McCollester's technique (Fig. 2) contain considerable amounts of plasma membranes in a sheet-like form but there is also some contamination with mitochondria and other cytoplasmic elements. Material derived from the normal lymphoid cells has a similar appearance. In view of the contamination of these fractions with intracellular organelles attempts were made to further purify the isolated plasma membranes by filtration through a column of glass beads. This technique has been described by Warren *et al.*[7] for the purification of chemically stabilized preparations of sheets of surface membranes from cultured murine cells and has also been successfully used by Hays and Barland[19] for the isolation of purified preparations of cell membranes from toad bladder epithelial cells. Material isolated by glass bead filtration was examined by electron microscopy and thin sections of these fractions are illustrated in Figs 3 and 4.

Membrane pellets produced from leukaemic lymphoblasts contain the same sheet-like membranes as demonstrated in Fig. 2, but the contaminating material has been removed by the filtration (Fig. 3). Membrane fractions derived from normal lymphoid cells after this further purification step also consist of a morphologically pure preparation of surface membrane (Fig. 4).

The membranes in these preparations may be contrasted with the vesicular fragments of plasma membrane obtained by the procedure of Wallach and Kamat[4] from normal lymphoid cells (Fig. 5). In this procedure fragments of plasma membrane are isolated from the top of the Ficoll or dextran barrier whilst vesicles derived from

Fig. 3. Typical field of a thin section of material obtained from the plasma membrane fraction of murine leukaemic cells by the method of McCollester[8] after further purification on a column of glass beads. Magnification × 25000. It can be seen that the fraction consists of sheets of plasma membrane. Note the absence of contaminating material.

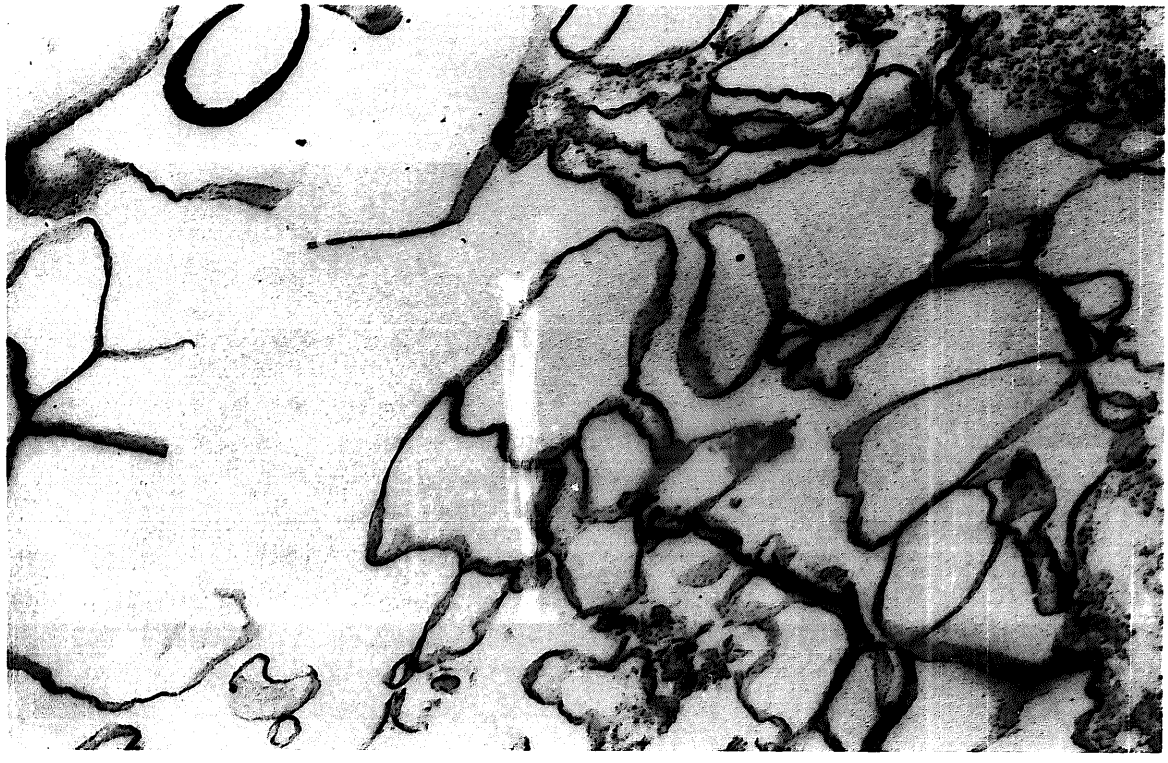

Fig. 4. Typical field of a thin section of material obtained from the plasma membrane fraction of normal lymphoid cells of mice by the method of McCollester[8] following purification on a column of glass beads. Magnification × 25000. The fraction consists of membrane sheets devoid of contaminating material.

Fig. 5. Typical field of a thin section of material obtained from the plasma membrane fraction of normal lymphoid cells of mice by the method Wallach and Kamat[4]. Magnification × 25000. The fraction which was obtained at a barrier of Ficoll (density 1.082) consists of numerous smooth vesicles.

the endoplasmic reticulum are deposited as a pellet at the base of the barrier. It will be appreciated that it is difficult to tell from the morphology of a vesicular preparation the membrane source from which the fragments are derived, and one must place more reliance on other means for assessing contamination.

Enzymatic analysis of membrane fractions

The level of $(Na^+ + K^+)$-stimulated ouabain-inhibited ATPase as a surface membrane marker was too low to be accurately determined in homogenates of leukaemic tissue, but vesicular fragments of the plasma membranes were shown to possess some activity (1.18 μmoles/h per mg protein) which was inhibited by 0.1 mM ouabain. The Mg^{2+}-dependent ATPase of vesicles derived from the surface membrane had a specific activity of 12.4 μmoles/h per mg protein compared to 3.5 μmoles/h per mg protein for the endoplasmic reticulum fragments. Homogenates and microsomes possessed specific activities of 1.91 ± 0.74 (6 batches) and 6.06 ± 0.86 (4 batches), respectively. The homogenate activity was inhibited 60% by 0.01 M ethacrynic acid, an inhibitor whose properties of the cation sensitive ATPase have been examined by Proverbio *et al.*[20]. In view of the low activities of these enzymes they were not employed further.

Data from the enzymatic analysis of a number of plasma membrane preparations are summarized in Table I. It should be pointed out that the scheme of preparation includes no conventional homogenization step from which material can be obtained for analysis to give a suitable baseline for assessment of purificaton in the isolation procedure. In order to overcome this problem an aliquot (usually 10^8 cells) of the cell suspension in the final washing step, before extraction, was taken, the cells pelleted at 500 × *g* for 5 min and the cells lysed in 1 ml of distilled water. This fraction is referred to as 'cell lysate' (Table I) and the enzymatic activities in this fraction were used as baseline activities. It was found that the specific activity of this fraction agreed with the level of 5′-nucleotidase activity found in the homogenate fraction when vesicular membrane preparations were prepared by the nitrogen cavitation method. As a control, cells were also lysed in 1 ml of extraction solution to give a manageable quantity of material for enzyme analysis. The enzymatic activity in this lysate was compared with that of a water lysate of the same batch of cells and it was found that with all the activities studied the specific activities in both lysates were identical within experimental limits, thus any loss of enzymatic activity within a fraction was not due to the conditions of extraction.

The activity of succinate dehydrogenase was either very low or undetectable in the final plasma membrane fraction in both normal and leukaemic lymphoid cells, showing that contamination of the purified plasma membranes by mitochondria is negligible, a finding in complete agreement with the morphological studies. As a marker for endoplasmic reticulum glucose-6-phosphatase has often been used, but with the cells under investigation the amounts of enzyme present were found to be too low to be estimated accurately. Ferber *et al.*[10] have suggested that NADH oxidoreductase may be used as a reliable marker of endoplasmic reticulum and is especially useful if glucose-6-phosphatase activity is low. In the 'pre-column' fraction, which is comparable to the plasma membrane preparation of McCollester[8], the specific activity of this marker was increased as compared to the cell lysate,

TABLE I

ENZYMATIC CHARACTERIZATION OF SUBCELLULAR FRACTIONS DERIVED FROM NORMAL AND LEUKAEMIC MURINE LYMPHOID CELLS

Specific activities are quoted as nmoles·(mg protein)$^{-1}$·min^{-1} and total activities as nmoles·min^{-1}.
The enzymes were assayed at 37 °C except in the case of NADH oxidoreductase which was determined at 20 °C. Values are given as means from triplicate determinations.

Normal cells	*5'-Nucleotidase*						*NADH oxidoreductase*			
	Preparation I		*Preparation II*		*Preparation III*		*Preparation IV**		*Preparation V**	
	Specific activity	*Total activity*	*Specific activity*	*Total activity*	*Specific activity*	*Total activity*	*Specific activity*	*Total activity*	*Specific activity*	*Total activity*
Cell lysate	8.4	578.5	45.0	958.5	28.5	600.4	9.5	30.8	6.1	57.6
Pre-column	33.7	56.6	25.5	97.6	43.3	59.8	18.5	1.0	26.0	2.2
Post-column	193.0	2.3	93.2	2.7	61.6	9.2	N.D.	N.D.	10.4	0.3

Leukaemic cells	*Preparation I*		*Preparation II*		*Preparation III*		*Preparation IV**	
	Specific activity	*Total activity*	*Specific activity*	*Total activity*	*Specific activity*	*Total activity*	*Specific activity*	*Total activity*
Cell lysate	102.7	290.1	56.4	680.5	40.6	926.1	18.2	400.3
Pre-column	120.4	46.8	56.4	45.6	89.9	158.4	63.2	59.8
Post-column	192.9	6.1	97.6	2.5	149.6	3.6	44.4	2.4

* Succinate dehydrogenase activity was investigated in these preparations and in the case of the normal cells although activity was found in the cell lysate (total activity 0.054 and 2.66 nmoles·min^{-1}) no detectable activity in the post-column fractions. In the case of the leukaemic tissue (total activity of cell lysate 5.78 nmoles·min^{-1}) negligible amounts were detected in the post-column fraction.
N.D., no enzymatic activity detectable.

although subsequent passage of the membrane enriched fraction through the bed of glass beads resulted in a removal of endoplasmic reticulum derived material, as judged by an elimination of activity or reduction in specific activity of NADH oxidoreductase, in fractions from normal lymphoid cells. In leukaemic cells one preparation (Preparation III) showed a small increase in specific activity and another (Preparation IV) a reduction in the specific activity of NADH oxidoreductase. These results may be contrasted with the level of NADH oxidoreductase found in a vesicular preparation of plasma membranes (normal: 114.7 nmoles/min per mg protein; leukaemic: 128.9 nmoles/min per mg protein) and endoplasmic reticulum (normal: 103 nmoles/min per mg protein; leukaemic: 231.4 nmoles/min per mg protein) derived from the same cell types. The similar levels of activity in the plasma membrane and endoplasmic reticulum fractions of the normal lymphoid cells is perhaps surprising. However, Ferber *et al.*[10] working with fragments of membrane derived from pig lymphocytes found the specific activity of NADH oxidoreductase of their plasma membrane fraction to be over twice that present in the fraction designated as endoplasmic reticulum.

5′-Nucleotidase is used by many authors as a marker enzyme for the plasma membrane. In normal lymphoid cells there is considerable lack of 5′-nucleotidase activity during the preparation of the plasma membrane fraction. Some 10% of the original activity is recovered in the 'pre-column' membrane enriched fraction and in turn between 3 and 16% of this activity is recovered in the 'post-column' fraction. Working with pig lymphocytes Allan and Crumpton[9] recovered 15% of the 5′-nucleotidase activity present in the original homogenate in their plasma membrane fraction, whilst Ferber *et al.*[10] recovered 50% of this enzyme in the microsomal fraction, and in a typical microsomal subfractionation found 80% of the available enzyme was recovered in the plasma membrane fraction. Although the recovery of 5′-nucleotidase in pig lymphocyte plasma membranes is considerably higher than in the murine cells examined by us, none the less it would appear that with the pig lymphocyte this enzyme is not an exclusive plasma membrane constituent, unless it is assumed that the activity found by Allan and Crumpton[9] to be distributed among all the subcellular fractions represents cross contamination by fragments of plasma membrane. In both the normal and malignant murine cells examined here the results are consistent with 5′-nucleotidase not being exclusively located within the plasma membrane. When the methods described by Ferber *et al.*[10] for isolating pig lymphocyte plasma membrane were applied to normal murine lymphoid cells, and the resulting fractions assayed for 5′-nucleotidase activity we found that 40% of the total activity was recovered in the nuclear-mitochondrial fraction and 1.7% in the plasma membrane fraction, a figure comparable with the recovery in Preparation III for normal lymphocytes. Morphological examination of the 'nuclear-mitochondrial' pellet in the electron microscope showed a lack of an appreciable amount of microsomal vesicles or unbroken cells and therefore it is concluded that in these cells a significant amount of 5′-nucleotidase activity is associated with the nucleus and/or mitochondria. An explanation for the low recovery of 5′-nucleotidase might be found in the well-documented reorientation of lymphocyte membrane components as a function of temperature, however, as the vesicular fragments are prepared at low temperature in contrast to the borate extraction procedure which is performed at room temperature and as

a low recovery of 5′-nucleotidase is obtained in both cases this may be unlikely.

It is interesting to note that Wallach and Ullrey[21] using histochemical techniques, found 5′-nucleotidase to be located exclusively in the nuclei of Ehrlich ascites carcinoma cells, and Gahmberg and Simons[22] working with BHK 21 cells were also able to show that there was little concentration of this enzyme during the isolation of plasma membranes.

Both the normal and the malignant lymphoid cells showed a wide variation in 5′-nucleotidase activity among different preparations. This result is identical with the findings of Allan and Crumpton[23] for human thymocyte plasma membrane preparations. Like these authors we are unable to find any relationship between activity or yield of plasma membrane. The consistent increase in specific activity of the 'post-column' fraction compared to the 'pre-column' fraction however, may well be indicative of a purification of plasma membrane associated enzyme.

Chemical analyses of isolated membrane

Although McCollester[8] prepared a plasma membrane fraction from the leukaemic cells of AKR mice, chemical analysis on this material was not reported. In the present investigation sufficient leukaemic tissue was available to enable chemical data on the plasma membrane of the malignant cells to be obtained and the results are summarized in Table II. A protein content of 51.8% dry weight and lipid content of 48.1% are well within the range expected for plasma membranes. The high value for the cholesterol phospholipid molar ratio (0.99) is considered from the work of Coleman and Finean[24] to indicate that the preparation consists mainly of plasma membrane and is in excellent agreement with the value of 1.01 and 1.03 reported by various authors[9,10] for pig lymphocyte plasma membranes.

TABLE II

CHEMICAL COMPOSITION OF PLASMA MEMBRANE FRACTION OF LEUKAEMIC CELLS

Values shown are mean (and standard deviation where appropriate) of the number of estimations shown in brackets.

Protein	(% dry wt)	51.8 ± 1.6	(6)
Total lipid	(% dry wt)	48.1	(1)
Phospholipid	(μg/mg of protein)	330	(2)
Cholesterol	(μg/mg of protein)	164	(2)
Cholesterol/phospholipid molar ratio		0.99	

Conclusions

Several methods have been described for the preparation of plasma membranes from various tissues, but only four methods have been described for the preparation of plasma membrane fractions from lymphocytes[9,10,25,26]. These workers have encountered similar difficulties in handling lymphocytes. One problem is that the use of sucrose causes unbroken lymphocytes to clump[10], and causes disintegration and aggregation of the nuclei[9] of broken cells. Another problem pointed out by Allan and Crumpton[9] is that when plasma membranes are produced in the vesicular

form, cytoplasmic components may be included in the vesicles which leads to difficulties in separation of the vesicles because of their heterogeneity with respect to both size and density. Separation of these vesicles into fractions composed of vesicles derived from smooth membrane which contain little contamination and vesicles which contain large amounts of contaminating material by equilibrium centrifugation involves long hours of centrifugation. Alternatively entrapped material may be removed by hypotonic shock as described by Ferber *et al.*[10] and this precautionary procedure was adopted in the preparation of vesicular fragments of plasma membranes in this work. With membrane ghosts isolated as open sheets the problem of entrapped soluble protein is minimised.

In the method which we have developed for the separation of plasma membrane ghosts not only is the problem of entrapped protein reduced but the use of sucrose at any stage is avoided. In addition the isolation method used, with the inclusion of the glass bead column purification step, results in a rapid method for the isolation of surface membranes allowing analytical investigations to be performed in the same day as the material was isolated.

Preliminary studies (performed at the Abteilung für Krebsforschung, University of Zurich) on the preparation of plasma membranes from the leukaemic L5222 myelomonocytic ascites cells of the BD IX rat, have shown that this method is also suitable for the preparation of a similar plasma membrane fraction from this source of cells.

The development of a rapid technique for isolating plasma membranes from normal and leukaemic cells provides not only a means whereby comparative studies on the surface properties of normal and malignant cells may be investigated further but the isolation of sheets of membrane enables topographical studies on cell surface components to be made.

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REFERENCES

1 Buck, C. A., Glick, M. C. and Warren, L. (1970) *Biochemistry* 9, 4567–4576
2 Emmelot, P. and Bos, C. J. (1972) *J. Membrane Biol.* 9, 83–104
3 Cook, G. M. W. and Jacobson, W. (1968) *Biochem. J.* 107, 549–557
4 Wallach, D. F. H. and Kamat, V. B. (1964) in *Methods in Enzymology* (Neufeld, E. and Ginsburg, V., eds), Vol 8, pp. 164–172, Academic Press, London and New York

5 Neville, Jr, D. M. (1960) *J. Biophys. Biochem. Cytol.* 8, 413–422
6 Emmelot, P., Bos, C. J., Benedetti, E. L. and Rümke, Ph. (1964) *Biochim. Biophys. Acta* 90, 126–145
7 Warren, L., Glick, M. C. and Nass, M. K. (1966) *J. Cell Physiol.* 68, 269–287
8 McCollester, D. L. (1970) *Cancer Res.* 30, 2832–2840
9 Allan, D. and Crumpton, M. J. (1970) *Biochem. J.* 120, 133–143
10 Ferber, E., Resch, K., Wallach, D. F. H. and Imm, W. (1972) *Biochim. Biophys. Acta* 266, 494–504
11 Hunter, M. J. and Commerford, S. L. (1961) *Biochim. Biophys. Acta* 47, 580–586
12 Persijn, J. P., van der Slick, W., Kramer, K. and de Ruijter, C. A. (1968) *Z. Klin. Chim.* 6, 441–446
13 Green D. E., Mü, S. and Kohout, P. M. (1955) *J. Biol. Chem.* 217, 551–567
14 Hubscher, G. and West, G. (1965) *Nature* 205, 799–800
15 Watson, D. (1960) *Clin. Chim. Acta* 5, 637–643
16 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375–400
17 Kopaczyk, K., Perdue, J. and Green, D. E. (1966) *Arch Biochem. Biophys.* 115, 215–225
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
19 Hays, R. M. and Barland, P. (1966) *J. Cell Biol.* 31, 209–214
20 Proverbio, F., Robinson, J. W. L. and Whittembury, G. (1970) *Biochim. Biophys. Acta* 211, 327–336
21 Wallach, D. F. H. and Ullrey, D. (1962) *Cancer Res.* 22, 228–234
22 Gahmberg, C. G. and Simons, K. (1970) *Acta. Path Microbiol. Scand.* section B 78, 176–182
23 Allan, D. and Crumpton, M. J. (1972) *Biochim. Biophys. Acta* 274, 22–27
24 Coleman, R. and Finean, J. B. (1966) *Biochim. Biophys. Acta* 125, 197–206
25 Demus, H. (1973) *Biochim. Biophys. Acta* 291, 93–106
26 Artzt, K. J., Sanford, B. H. and Marfey, P. S. (1968) *J. Lab. Clin. Med.* 72, 350–352