Biochimica et Biophysica Acta, 311 (1973) 173–179

BBA 76366

A RAPID METHOD FOR THE ISOLATION OF RAT LIVER PLASMA MEMBRANES USING AN AQUEOUS TWO-PHASE POLYMER SYSTEM

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(Received February 13th, 1973)

SUMMARY

A simple and rapid method for the isolation of rat liver plasma membranes has been developed using an aqueous two-phase polymer (dextran–polyethyleneglycol system). Yields and purity of this preparation were determined and compared to membranes obtained by sucrose density gradient centrifugation. The technique as described in this paper provides:

1. The highest reported yield of plasma membrane when compared to other methods.

2. A high degree of purity determined by enzyme assays of the plasma membrane fraction, that is comparable to other separation techniques.

3. A simple and rapid method, requiring only low speed centrifugations, decreasing membrane preparation time by 2–3 h.

INTRODUCTION

Fractionation methods developed for the isolation and purification of liver plasma membranes have previously relied exclusively on density separation techniques. These "classical" methods have been recently summarized by Hinton[1]. While these methods have provided satisfactory yields and purity, they may lead to structural damage caused by sedimentation through solutions having a high interfacial tension[2]. The stabilizing effect of aqueous solutions of polymers on whole cells[3], chloroplasts[4], viruses[5] and enzymes[6] has been well documented[7]. Recently an aqueous two-phase polymer (dextran–polyethyleneglycol) system has been applied to the isolation of plasma membranes from cells grown in suspension culture[8].

The purpose of this study was to develop an aqueous two-polymer phase system for the rapid isolation and purification of the plasma membranes from adult rat liver. The present paper describes a study whereby comparisons are made between plasma membrane preparations isolated by conventional sucrose density gradient techniques and those isolated by differences in membrane surface characteristics in a liquid–liquid polymer system. We have examined the purity of these preparations by the enzyme markers 5′-mononucleotidase, Mg^{2+}-stimulated ATPase, (Na^+-K^+)-

Abbreviation: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

activated ATPase, glucose 6-phosphatase, and succinate dehydrogenase, and compared the yields obtained using these two procedures.

MATERIALS AND METHODS

Preparation of the polymers

Stock solutions of the two phase system (dextran 500 and polyethyleneglycol 6000) were prepared as previously described by Brunette and Till[8] with the following modifications: $ZnCl_2$ (10^{-2} M) was omitted and replaced by 80 ml of distilled water. Stock solutions of the two phase system were prepared in the following manner and stored at 5 °C: 200 g of 20% (w/w) Dextran 500 (Pharmacia Fine Chemicals, Sweden) in distilled water, 103 g of 30% (w/w) polyethylene-glycol (Carbowax 6000, Matheson, Coleman and Bell, Norwood, Ohio), 333 ml of 0.22 M phosphate buffer (sodium salts) at pH 6.5, and 179 ml of distilled water were mixed in a separatory funnel and allowed to settle for 48 h in the cold. The two phases were collected.

Preparation of rat liver plasma membranes

Plasma membranes were isolated from the livers of male Sprague–Dawley rats (Blue Spruce Farms, Altamont, N.Y.), fed *ad libitum*. The rats weighing 120–170 g were sacrificed by decapitation, and a 7.0-g portion of the liver removed and washed in chilled 0.5 mM $CaCl_2$ containing 1.0 mM $NaHCO_3$ medium (pH 7.5). All subsequent steps in the purification procedure were performed at 5 °C. Following the method originally described by Ray[9], a low spin ($1000 \times g$) pellet was obtained. This pellet was equally divided and prepared for either sucrose density or aqueous polymer centrifugation. Therefore, membranes obtained from the same liver were purified by each method and subsequently used in assaying for membrane purity.

The sucrose density centrifugation followed the procedure described by Ray[9] which has been shown to yield a highly purified plasma membrane fraction as demonstrated by enzyme markers, chemical analysis and electron microscopy. Briefly, the low-speed pellet was suspended in a discontinuous sucrose gradient and centrifuged for 2 h at $65\,000 \times g$ ($25 \cdot 10^3$ rev./min) in a Spinco SW-25.1 rotor in a Beckman Model L centrifuge. The purified plasma membrane fraction banding between the 37 and 41% sucrose layers was removed with a Pasteur pipette.

The second half of the low-speed pellet was suspended in 30 ml of the top phase of the two polymer mixture. 10-ml aliquots were pipetted into three 50-ml Sorvall polycarbonate centrifuge tubes. To each tube was added 10 ml of bottom phase of the two polymer mixture. The tubes were gently shaken and centrifuged for 15 min at $1100 \times g$ (3000 rev./min) in an International Equipment centrifuge (IEC) Model PR-2, (International Equipment Co., Boston, Mass.) equipped with rotor No. 269 at 4 °C. The plasma membrane, which formed a thin white sheet at the interface between the two polymers was removed with a Pasteur pipette and resuspended in 10 ml of fresh top phase of the two polymer mixture in a clean tube. The centrifugation procedure described above was repeated a total of 3 times. Purified plasma membranes located at the interface were obtained after a total centrifugation time of 45 min.

The plasma membrane suspensions prepared by sucrose density and aqueous polymer centrifugations were washed in the following manner: 25 ml of 50 mM

Tris buffer (pH 7.5) were added to each membrane preparation. The diluted suspensions were centrifuged for 10 min at 2400 × *g* (5500 rev./min) in an IEC-PR-2 centrifuge with a No. 296 rotor. The supernatants were discarded and the pellets were resuspended by homogenization and washed with three successive 30-ml portions of Tris buffer. The final pellets from both preparations were suspended in 50 mM Tris buffer (pH 7.5) and/or 0.1 M sodium acetate buffer (pH 6.1) depending on the enzyme marker assay that was performed.

Protein was determined by the method of Lowry *et al.*[10] with bovine serum albumin (Fraction V, Sigma Co., St. Louis, Mo.) dissolved in buffer as the standard.

Enzyme assays for evaluating membrane purity

Mg^{2+}-stimulated ATPase (EC 3.6.1.3). Mg^{2+}-stimulated ATPase was assayed at pH 7.5 using ATP as substrate. The assay system contained in a final volume of 1.0 ml, 50 mM Tris buffer, 10 mM $MgCl_2$, 5 mM disodium salt of ATP (Sigma Chemical Co., St. Louis, Mo.) and membrane suspension (100–130 μg protein). After incubation at 37 °C for 30 min the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. 1-ml aliquots of the deproteinized supernatants were analyzed for P_i. The amount of P_i released was determined colorimetrically by the method of Harris and Popat[11]. Control tubes containing membrane only and substrate only were subtracted from the experimental values.

(Na^+–K^+)-activated ATPase (EC 3.6.1.4). (Na^+–K^+)-activated ATPase was assayed using a modified method of Brunette and Till[8]. The assay buffer, 0.05 M Tris buffer (pH 7.5), contained 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA). The specific activity of this enzyme was calculated from an increase in the amount of P_i released which occurred when K^+ was added to the assay system which already contained Na^+.

5'-Mononucleotidase (EC 3.1.3.5). 5'-Mononucleotidase was assayed at pH 7.5 using 5'-AMP as substrate. The assay system contained in a final volume of 1.0 ml, 50 mM Tris buffer, 10 mM $MgCl_2$, 5 mM 5'-AMP and membrane protein 100–130 μg. After a 30-min incubation period at 37 °C, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. 1-ml aliquots of the deproteinized supernatants were assayed for the amount of phosphate released.

Glucose-6-phosphatase (EC 3.1.3.9). Glucose-6-phosphatase was assayed according to the method of Hübscher *et al.*[12], using 0.1 M sodium acetate buffer (pH 6.1). The P_i liberated after a 15-min incubation at 30 °C was determined.

Succinate dehydrogenase (EC 1.3.99.1) was determined spectrophotometrically by the method of King[13].

RESULTS

Isolation of the purified membrane fraction

Separation of subcellular components by the aqueous polymer technique has been recently applied to the purification of plasma membranes. The adaptation of this technique to the separation of rat liver plasma membrane required several modifications. The membrane material located at the interface between the two polymers after the first centrifugation, is transferred to new polymer solution at each subsequent centrifugation step. All centrifugations are performed at low speeds

(*i.e.* $1100 \times g$) thereby eliminating the need of expensive high speed centrifugation equipment. An important point that must be emphasized is the necessity for careful and repeated washing of the membrane to remove adsorbed polymer. The polyethyleneglycol interferes with the colorimetric development in assays used to determine amounts of protein and phosphate[14]. If the polymer is not removed, high phosphate values will appear in the membrane blank.

Yield of membrane

The properties of the membrane preparations for each method are shown in Table I. The yields are expressed as mg protein per g of wet wt of liver tissue. It is apparent from these results that a consistent increase in yield is observed in each of the three experiments where the membrane material was prepared by polymer (dextran–polyethyleneglycol) centrifugation. This is also evident from the average values shown in parenthesis.

TABLE I

YIELD AND PURITY OF RAT LIVER PLASMA MEMBRANE SEPARATED BY SUCROSE GRADIENT AND AQUEOUS POLYMER CENTRIFUGATION

Adult rat liver plasma memebranes were prepared by sucrose density gradient centrifugation and a two-phase aqueous (dextran–polyethyleneglycol) polymer system. Comparative results of the two methods of preparation showing the yield and specific activity of plasma membrane markers can be seen below. The yield is expressed as mg of membrane protein per g wet wt of liver. The enzyme activities expressed as μmoles phosphate released per mg protein per 30 min were determined as described in Materials and Methods. Each experiment contained triplicate enzyme analysis, and the results are given as the mean $\pm$S.E. The numbers shown in the parentheses represent the mean $\pm$S.E. of the three experiments.

	Expt No.	*Methods of plasma membrane preparation*	
		Two phase system	*Sucrose density method*
Yield	I	2.71	2.22
	II	3.17	2.42
	III	2.20	1.34
		(2.69 $\pm$ 0.28)	(1.99 $\pm$ 0.33)
Marker enzyme activity			
Mg^{2+}-stimulated ATPase	I	15.43 $\pm$ 0.90	16.59 $\pm$ 0.94
	II	24.09 $\pm$ 0.87	17.79 $\pm$ 1.73
	III	22.17 $\pm$ 0.45	27.13 $\pm$ 0.85
		(20.56 $\pm$ 2.62)	(20.49 $\pm$ 3.32)
(Na^+-K^+)-activated ATPase	I	2.14 $\pm$ 0.61	2.66 $\pm$ 1.53
	II	2.96 $\pm$ 0.60	3.00 $\pm$ 0.82
	III	2.50 $\pm$ 0.15	2.20 $\pm$ 0.95
		(2.53 $\pm$ 0.24)	(2.62 $\pm$ 0.23)
5′-mononucleotidase	I	36.13 $\pm$ 0.39	36.66 $\pm$ 1.65
	II	25.33 $\pm$ 0.23	23.16 $\pm$ 0.42
	III	23.68 $\pm$ 0.68	29.61 $\pm$ 1.10
		(28.38 $\pm$ 3.90)	(29.81 $\pm$ 3.90)

Enzymatic criteria for membrane purity

The purity of the plasma membrane preparations may be evaluated by the measurement of marker enzymes shown to be characteristic of a particular subcellular component. Mg^{2+}-stimulated ATPase, $(Na^{+}-K^{+})$-activated ATPase and 5'-mononucleotidase activities were chosen as standard markers for plasma membranes. Results from three separate experiments are shown in Table I. A remarkable similarity between the two methods of preparation in respect to the level of these marker enzymes is apparent. Our values for Mg^{2+}-ATPase are in agreement with those of Emmelot *et al.*[15] but lower than those reported by Ray[9]. If one does not subtract the ATP blank in the determination of liberated P_i, erroneous high values for ATPase will be obtained.

The degree of contamination between the two membrane preparations by other subcellular components (*e.g.* endoplasmic reticulum and mitochondria) were evaluated by enzyme markers for these particles. The results are shown in Table II. It is evident that the enzyme contamination in the plasma membranes prepared by the polymer method was no greater than those prepared by the standard sucrose gradient method. The considerably low activity of glucose-6-phosphatase in the plasma membrane, (0.65 ± 0.05 μmole per mg membrane protein per 30 min) suggests minimal contamination when compared to the activity of the microsomal bound enzyme (11.0 μmoles per mg membrane protein per 30 min) as reported by Ray[9]. The data may suggest contamination of the membrane preparation by smooth microsomes to the extent of 5–6%.

TABLE II

ASSESSMENT OF CONTAMINATION OF PLASMA MEMBRANE PREPARATIONS BY SUBCELLULAR MEMBRANES AS DETERMINED BY ENZYME MARKERS

Purified plasma membranes were prepared as described in Materials and Methods. Contamination by endoplasmic reticulum was determined by measurement of glucose-6-phosphatase. Specific activity is expressed as μmoles of phosphate released per mg protein per 30 min. The degree of mitochondrial contamination was evaluated by the measurement of succinate dehydrogenase as described in Materials and Methods. The specific activity is recorded as μmoles substrate utilized per mg protein per min. The values obtained from purified membranes by two methods are compared. The results are given as the mean ± S.E. The numbers shown in parentheses represent the mean ± S.E. for the three separate experiments.

Enzyme activity	*Expt. No.*	*Methods of plasma membrane preparation*	
		Two phase system	*Sucrose density method*
Succinate dehydrogenase	I	0.010 ± 0.000	0.013 ± 0.001
	II	0.010 ± 0.001	0.011 ± 0.000
	III	0.018 ± 0.001	0.010 ± 0.001
		(0.013 ± 0.003)	(0.011 ± 0.001)
Glucose-6-phosphatase	I	0.72 ± 0.01	0.62*
	II	0.65 ± 0.02	
	III	0.55 ± 0.09	
		(0.64 ± 0.05)	

* Mean of 20 determinations, standard error values not stated (ref. 9).

DISCUSSION

In our studies which involve the adaptation of the aqueous polymer (dextran–polyethyleneglycol) system to the isolation of the rat liver plasma membrane, we have demonstrated a significant increase in the yield of the product. This increase in the yield represents 49% more membrane material than the method described by Ray, without compromising purity of the preparation. The results clearly show that contamination by other cell components cannot explain the difference in the yield between the two methods of preparation. The loss of membrane material in the sucrose density gradient method can be accounted for by membrane material present in nuclei sediment and at each band in the discontinuous gradient as determined by 5′-mononucleotidase activity (Sohn, R., unpublished).

For effective purification it is important that the aqueous polymer system is not overloaded with excess low speed pellet material. Limiting the starting material to 7.0 g wet wt of tissue results in adequate purification of the plasma membrane.

Phase contrast microscopy was used to monitor plasma membrane preparations at various stages of their purifications. This was possible because plasma membranes obtained by aqueous polymer centrifugation were less fragmented than membranes purified by sucrose density centrifugation. The nuclei and cell debris remained in the bottom phase and frequently a pellet of these was formed.

We suggest that the two phase (dextran–polyethyleneglycol) system described in this communication can be used with advantage in the preparation of rat liver plasma membrane. This method of purification offers the largest reported yield of plasma membrane with equivalent purity when compared to membranes purified by standard sucrose density methods. In addition, the technique is simple, rapid, and required only low speed centrifugations. The membrane obtained from this procedure has a high degree of purity, as assessed by enzyme markers, and is physically less fragmented (as observed by phase microscopy) than membranes obtained by sucrose density gradient separation.

ACKNOWLEDGMENTS

This investigation was supported in part by Grants HL 02063 from The Heart and Lung Institute, National Institutes of Health GRS6 Institutional Sub-Award (G. V. Marinetti and L. Lesko) and grant CA-07239 from The National Cancer Institute (J. D. Hare and M. Donlon).

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