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ISOLATION OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM FRAGMENTS FROM CHICK EMBRYO FIBROBLASTS

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SUMMARY

Plasma membrane and endoplasmic reticulum were isolated from chick embryo fibroblasts using a derivation of the method of Avruch and Wallach (*Biochim. Biophys. Acta*, 233 (1971) 334). The fractions isolated were checked for purity using 5′-nucleotidase and (Na^+, K^+, Mg^{2+})-ATPase as markers for plasma membrane, NADPH:cytochrome *c* reductase as a marker for endoplasmic reticulum and succinate dehydrogenase as a marker for mitochondrial contamination. The method yielded a plasma membrane fraction containing less than 4 % of the endoplasmic reticulum and an endoplasmic reticulum fraction containing less than 2 % of the plasma membrane. Mitochondrial contamination of both fractions was less than 3%. Similar yields and purity were obtained for fractions isolated from cells which had been infected with either Semliki Forest virus or with Newcastle Disease virus.

INTRODUCTION

Studies on the replicative cycle of animal viruses include the use of cell fractionation techniques to determine the intracellular location of sub-viral components or to detect changes in the host cell structure during the course of the infection. We are particularly interested in a method for obtaining both the plasma membrane and endoplasmic reticulum from chick embryo fibroblasts. A variety of methods are available for isolating the plasma membrane only from several different types of cell[2–5], and for isolating both plasma membrane and endoplasmic reticulum from Ehrlich ascites carcinoma cells[6], HeLa cells[7], and rat fat cells[1]. However, for chick embryo cells only the isolation of the plasma membrane without the concurrent isolation of endoplasmic reticulum has been described[8,9].

Preliminary experiments using a modification of the Tris method of Warren *et al.*[3] proved unsuccessful with the chick cells, although excellent preparations of plasma membranes were obtained from L929 and BHK21/C13 cells by the same method. We therefore tried the method of Avruch and Wallach[1] which exploits the difference in isopycnic density of the two types of membrane vesicles under conditions of low ionic strength. Our method avoids the use of their sucrose gradient step which we found to give a much lower yield of endoplasmic reticulum recovered in the Dextran gradient.

METHODS AND RESULTS

Materials

Dextran T40 was obtained from Pharmacia Ltd., Uppsala, Sweden. Cytochrome *c* (oxidised form), ATP, AMP and Tris base were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and NADPH from C. F. Boehringer und Soehne GmbH, Mannheim, Germany. [2-^{3}H]AMP and [γ-^{32}P]ATP were purchased from the Radiochemical Centre, Amersham, Bucks., G.B. All water used throughout was glass distilled after deionization.

Enzymic determinations

NADPH:cytochrome *c* reductase was estimated by the method of Ragnotti *et al.*[10]. Succinate dehydrogenase activity was measured by the method of Porteous and Clark[11]. (Na^+, K^+, Mg^{2+})-ATPase and 5'-nucleotidase were both estimated by the method of Avruch and Wallach[1]. Protein estimations for determination of specific activities were performed by the method of Lowry *et al.*[12].

Cell isolation and fractionation

Chick cells grown in monolayers on glass petri dishes (M. J. Morser, S. I. T. Kennedy and D. C. Burke, unpublished) were washed once on the plate with ice-cold sucrose–Tris–HCl–magnesium acetate buffer (0.25 M sucrose–50 mM Tris–HCl (pH 7.4)–1 mM magnesium acetate), scraped off with a rubber policeman and suspended in the same buffer in 20-ml aliquots containing $6 \cdot 10^8$–$10 \cdot 10^8$ cells. These and all subsequent operations were performed at 0–4 °C. The suspended cells were pelleted by sedimentation for 5 min at 1500 × *g*, the pellet from each 20-ml aliquot being resuspended in 5 ml of fresh sucrose–Tris–HCl–magnesium acetate buffer and homogenised with a tight-fitting glass Dounce homogeniser (20 strokes). Under these conditions over 90 % of the cells appeared to be disrupted without visible damage to the nuclei when examined by phase contrast microscopy. Initially two other buffers were tried: 50 mM Tris–HCl (pH 7.4)–1 mM magnesium acetate and 0.25 M sucrose–5 mM Tris–HCl (pH 7.4)[1], but neither of these gave a homogenate as reproducible or homodisperse as the sucrose–Tris–HCl–magnesium acetate buffer finally chosen.

In a preliminary experiment, the homogenate was layered on a linear 25–45 % (w/w) sucrose gradient and centrifuged for 90 min at 65000 × *g* as described by Avruch and Wallach[1]. Bands were formed in the gradient similar to those described; however, 48 % of the total cellular NADPH:cytochrome *c* reductase was found to be in the lower band (designated mitochondrial zone by Avruch and Wallach), 50 % in the pellet and none was detectable in the upper layer (their microsomal zone). The experiment was repeated using a discontinuous sucrose gradient (7 ml 45 % (w/w)–9 ml 25 % (w/w) sucrose and 5 ml homogenate) in place of the linear gradient, all other conditions being unchanged. This gave sharper bands; however, the distribution of the endoplasmic reticulum marker enzyme was similar to the first experiment. It was therefore decided that this method was unsuitable for chick cells, and the following procedure was subsequently used.

The homogenate was centrifuged for 10 min at 4000 × *g* in a MSE Highspeed 18 centrifuge, the pellet rehomogenised in a further 5 ml of sucrose–Tris–HCl–magnesium acetate buffer and resedimented at 4000 × *g* as above. The supernatant fractions from

the two 4000 × *g* centrifugations were pooled and recentrifuged at 4000 × *g* for 10 min. Sedimentation at 4000 × *g* resulted in 92 % of the mitochondria (indicated by estimation of succinate dehydrogenase) being pelleted, wheras 29 % of the total endoplasmic reticulum marker enzyme, NADPH:cytochrome *c* reductase was in the pellet. Increasing the centrifugal force to 92000 × *g* caused 100 % of the total mitochondrial enzymic activity to be sedimented. However, since 58 % of the endoplasmic reticulum marker was also deposited, the lower centrifugation speed was used routinely.

The supernatant from the final 4000 × *g* sedimentation was then centrifuged at 104000 × *g* for 2 h in a MSE Superspeed 50 centrifuge and the pellet resuspended by stirring with a glass rod in 4 ml of 1 mM Tris–HCl, pH 8.6, containing either 0.5 mM, 1.0 mM, 3.0 mM or 5.0 mM magnesium acetate. The resuspended material was then dialysed for 2 h against 2 × 1 l of the buffer used for resuspension. After dialysis, the samples were placed on gradients of Dextran T40 dissolved in the same buffer, composed as in Fig. 1a. The gradients were centrifuged at 82000 × *g* overnight (12–18 h) in a MSE Superspeed 50 centrifuge, and the membrane bands formed are depicted in Figs 1b–1e. It will be seen that up to three bands, designated T (top), M (middle) and B (bottom) were formed. The bands were collected by aspiration with a Pasteur pipette and assayed for the marker enzymes 5′-nucleotidase, (Na^+,K^+,Mg^{2+})-ATPase succinate dehydrogenase and NADPH:cytochrome *c* reductase. The results of the estimations are given in Table I.

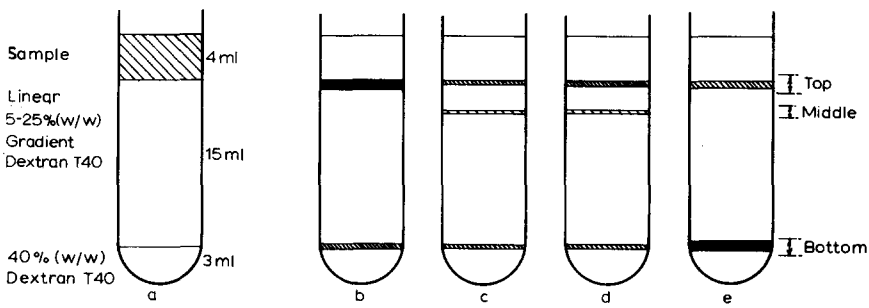

Fig. 1. Dextran T40 gradients. Tube a: gradient prepared before centrifugation; Tubes b–e: after centrifugation. b, gradient containing 0.5 mM Mg^{2+}; c, gradient containing 1.0 mM Mg^{2+}; d, gradient containing 3.0 mM Mg^{2+}; e, gradient containing 5.0 mM Mg^{2+}.

DISCUSSION

Assuming 5′-nucleotidase and (Na^+,K^+,Mg^{2+})-ATPase to be specific markers for the plasma membrane[1,7], and NADPH:cytochrome *c* reductase for endoplasmic reticulum[10,13], the best separation of the two types of membrane occurs in the gradient containing 0.5 mM Mg^{2+}. With increasing Mg^{2+} concentration there is an increasing accumulation of plasma membrane in the lower bands of the gradient. The middle band, M, occasionally appears as a minor component in the 0.5-mM Mg^{2+} gradient; in such cases it carries a similar enzymic distribution to the top layer, T. The chick cell (Na^+,K^+,Mg^{2+})-ATPase was a rather unsatisfactory marker for plasma membrane; the levels of ATPase in the whole cell not stimulated by Na^+ + K^+ are comparatively high making measurement of the slight stimulation subject to a high level

TABLE I

DISTRIBUTION OF MARKER ENZYMES IN VARIOUS FRACTIONS RECOVERED DURING MEMBRANE ISOLATION PROCEDURE

1 unit = 1 nmole substrate utilised/min. Where appropriate results are given as ± S.E. —, not measured. T, M and B are the top, middle and bottom layers, respectively, collected from the gradients depicted in Fig. 1. Figures in parentheses under heading *Fraction* indicate number of experiments upon which enzymic estimations were performed.

Fraction	*5'-Nucleotidase*		*(Na^+, K^+, Mg^{2+})-ATPase*		*NADPH:cytochrome c reductase*		*Succinate dehydrogenase*	
	Units/mg protein	*Total recovered (%)*	*Units/mg protein*	*Total recovered (%)*	*Units/mg protein*	*Total recovered (%)*	*Units/mg protein*	*Total recovered (%)*
Homogenate (3)	2.26 ± 0.3	(100)	1.86 ± 1.2	(100)	2.88 ± 0.4	(100)	11 ± 0.2	(100)
4000 × *g* pellet (3)	2.83 ± 0.4	50 ± 6	—	—	6.9 ± 0.8	29 ± 4	35 ± 0.3	92 ± 2
0.5 mM Mg^{2+} (3)								
T	11.4 ± 0.8	21 ± 2	72.5 ± 6.5	27 ± 10	0	0	0	0
B	2.1 ± 0.4	2 ± 1	0	0	25.0 ± 1.3	27 ± 5	0	0
1.0 mM Mg^{2+} (1)								
T	8.7	17	—	—	0	0	0	0
M	2.4	5	—	—	13.2	6	0	0
B	2.3	4	—	—	21.1	31	0	0
3.0 mM Mg^{2+} (1)								
T	3.8	8	—	—	0	0	0	0
M	1.3	3	—	—	16.2	5	0	0
B	8.3	16	—	—	13.7	34	0	0
5.0 mM Mg^{2+} (1)								
T	1.7	3	31.6	11	0	0	0	0
B	10.3	20	22.2	16	18.6	30	0	0

of error. Therefore, we used 5′-nucleotidase, which occurs in reasonable levels in chick cells, as the main marker for plasma membrane. Hence we deduce that the endoplasmic reticulum fraction (B) contained 2% contamination by plasma membrane. Although none of the mitochondrial marker, succinate dehydrogenase, was detectable in any of the membrane fractions, the sensitivity of the assay is such that an enzyme level less than 3% of the total found in the homogenate would not be estimable. Hence it is possible that the isolated membrane fractions may contain mitochondrial contamination to a maximum extent of 3% of the total cellular content. Similar reasoning applies to the non-detection of NADPH:cytochrome *c* reductase in the top layer fractions of the gradients. In this case a maximum of 4% endoplasmic reticulum contamination could escape detection.

These levels of enzyme recovery in the different fractions, if truly representative of the amounts of the appropriate membrane present, indicate a much higher purity of the recovered membranes than that reported for rat fat cells[1]. However, it is not possible to tell whether the improved purity of the fractions is attributable to the difference in the two isolation methods, or to the difference in the two cell types.

To test the applicability of the method for isolating the membrane fractions from virus infected cells, the above procedure (using 0.5 mM Mg^{2+} in the dialysis and gradient) was carried out on chick cells which had been incubated in the presence of 1 μg/ml actinomycin D for 3 h before infection and infected with either Newcastle Disease virus (strain Texas) or with Semliki Forest virus, the cells being harvested 8 h after infection in the former case, and 6 h in the latter case. Similar membrane bands and similar enzymic levels were found in comparison to the results with the uninfected cells. Studies on the incorporation of virus induced proteins into the membranes will be published elsewhere.

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