Microsomal membrane subfractionation by a lectin affinity method

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Concanavalin A-agarose treatment of rat liver post-mitochondrial supernatant removes a fraction rich in cholesterol and 5'-nucleotidase activity but low in glucose-6-phosphatase. At the same time, radiolabel associated with the cell surface is removed. We interpret these findings as evidence that concanavalin A binds to, and under these circumstances will remove, fragments of plasma membrane present in the microsomal fraction and believe that this may be of use in the gentle, and rapid subfractionation of microsomal membranes.

Concanavalin A *Immobilized lectin* *Microsomal subfractionation* *Plasma membrane*

1. INTRODUCTION

Microsomal membrane preparations obtained by centrifugation of post-mitochondrial supernatants contain membranes derived from a variety of cell organelles; although mostly derived from endoplasmic reticulum, fragments of plasma membrane and the Golgi system are also present (e.g. [1]). Conventional methods of microsomal membrane subfractionation depend upon centrifugation in high gravitational fields for long periods, conditions known to damage the constituent membrane species [2,3]. A previous report has, however, suggested that immobilized lectins could be used to subfractionate microsomal preparations as part of a gentle, rapid scheme of membrane subfractionation [4]. Here, we describe a lectin affinity method that removes a subfraction from microsomal preparations that is identified as plasma membrane.

Upon homogenization, cellular membrane systems vesicularize with a particular, characteristic asymmetry. Plasma membranes, for example, expose their 'extracellular', carbohydrate-rich surfaces to the medium whereas vesicles derived from the endoplasmic reticulum expose their 'cytosolic' surface to the medium, their carbohydrate residues being enclosed within the membrane lumen [5]. The lectin concanavalin A will bind to the surface of the parenchymal cells of rat liver [6] and therefore we have investigated the ability of this lectin to abstract fragments of plasma membrane 'contaminating' microsomal membrane preparations.

2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase (type IV, *Clostridium histolyticum*) was purchased from Sigma (Poole, England), agarose-bound concanavalin A (6 mg per ml settled gel) from BDH (Poole, England), and Bio-gel A150m from Bio-Rad (Watford, England). [U-^{14}C]Aniline (50 mCi/mmol) was purchased from Amersham (Aylesbury, England) and used for the synthesis of [U-^{14}C]sulphanilic acid (17×10^6 dpm/mmol) by the method of Vogel [7].

2.2. *Chemical estimations*

Protein was estimated by the method of Lowry

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et al. [8] and RNA by that of Fleck and Begg [9].

Phospholipid was extracted from samples according to Folch et al. [10]. Aliquots of phospholipid extract were evaporated to dryness in a water bath under nitrogen, and then digested until colourless with 0.4 ml perchloric acid (72%). The resulting phosphate was estimated by the method of Fiske and SubbaRow [11].

Cholesterol was estimated by the method of Moore et al. [12]. For the estimation of total (i.e. esterified and non-esterified) cholesterol, the samples were first saponified and non-saponifiable lipid extracts used for the sterol estimations. Membrane samples (4–6 mg protein) were incubated overnight at room temperature with methanolic KOH (5 ml of 15% (w/v) KOH). This was then saponified at 70°C for 1½ h and non-saponifiable lipid extracted 3 times with 10 ml petroleum spirit (b.p. 40–60°C). The extract was evaporated to dryness in a water bath under a stream of nitrogen before cholesterol estimation.

2.3. *Enzyme assays*

Glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of Ferreira [13]. 100 μl of sample (containing 1–10 mg protein) was added to 0.9 ml incubation mixture containing 20 μmol glucose 6-phosphate and 60 μmol sodium cacodylate, pH 6.5, after preincubation for 10 min at 37°C. The reaction was stopped after 10 min at 37°C by the addition of 1 ml ice-cold 10% (w/v) trichloroacetic acid. Blanks without added substrate and without sample were included. After centrifugation at 1000 × g_{av} for 10 min, 1 ml aliquots were removed for phosphate determination as in [11].

5′-Nucleotidase (EC 3.1.3.5) was assayed according to [14]. Occasional leaching of concanavalin A from the agarose gel inhibited 5′-nucleotidase activity. This inhibition may be relieved, however, by α-methyl mannoside and therefore assays were routinely performed in the presence and absence of 0.1 and 0.2 M α-methyl mannoside. Only assays in which the measured activities in 0.1 and 0.2 M α-methyl mannoside were the same have been considered for this work.

Ouabain-sensitive ATPase was assayed as follows: to a final volume of 1 ml containing 0.25 M sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.2 mM EGTA, 100 mM NaCl, 20 mM KCl and 4 mM ATP, pH 7.4, was added 50 μl of sample (containing 0.5–5 mg protein) and incubated in the presence and absence of 4 mM ouabain at 37°C. The reaction was stopped at various time intervals by the addition of 1 ml ice-cold trichloroacetic acid (10%, w/v). After centrifugation at 3000 × g_{av} at 4°C for 10 min, 1 ml aliquots of the resulting supernatant were removed for phosphate determination as described in [15].

Over the range of activities measured, activity was directly proportional to the concentration of sample protein assayed for each of these 3 enzyme assays – glucose-6-phosphatase, 5′-nucleotidase and ouabain-sensitive ATPase.

2.4. *Tissue preparations and treatments*

The livers of male Sprague-Dawley rats (fed at libitum, body wt approx. 200 g) were perfused with ice-cold saline (0.9% (w/v) NaCl) to remove blood before excision and homogenization with 2.5 vols of 20 mM Hepes, pH 7.5, containing 25 mM KCl and 5 mM $MgCl_2$. The homogenate was immediately centrifuged at 12000 × g_{av} for 20 min. The resulting post-mitochondrial supernatant was incubated with washed concanavalin A-agarose (1 ml post-mitochondrial supernatant to 2 ml settled gel containing 12 mg bound lectin) for 30 min at 7°C. The gel was then poured into a sintered glass tube and washed with the Hepes buffer until no more protein was eluted. This normally resulted in a dilution of the original post-mitochondrial supernatant components by 3–4-fold. A greater proportion of gel to supernatant resulted in a greater loss of both endoplasmic and plasma membrane markers, and in excessive dilution.

Hepatocytes were prepared by a modification of the method of Howard and Peach [15]. The livers of male Sprague-Dawley rats were perfused in situ with 20 ml collagenase (75 mg/100 ml in Hank's medium) to remove blood, via the hepatic portal vein. The liver was removed and sliced thinly by hand, then incubated in 150 ml collagenase (as above but supplemented by 2% (w/v) albumin) for 30 min at 37°C with oxygenation. The resulting cell suspension was pushed through boulting cloth (100 μm size). Parenchymal cells were recovered from filtered cell suspensions by centrifugation at 50 × g for 3 min and resuspended in Hank's medium (30 ml) for incubation with diazotized [^{14}C]sulphanilic acid (3.5 mM). Diazotization of sulphanilic acid was carried out according to [16].

Table 1

Percent recoveries of various membrane markers after filtration of post-mitochondrial supernatant through concanavalin A-agarose or agarose gel (Biogel A150 m)

	Concanavalin A	Agarose
Protein	96 ± 2(2)	–
RNA	98 ± 2(2)	–
Total cholesterol	69 ± 3(6)	–
Free cholesterol	79 ± 4(8)	–
Phospholipid	87 ± 5(3)	88 ± 3(2)
Glucose-6-phosphatase	99 ± 2(9)	96 ± 3(4)
5′-Nucleotidase	41 ± 5(4)	94 ± 4(4)

Values are percent recovery of activity loaded ± SE (number of separate experiments in parentheses)

3. RESULTS AND DISCUSSION

The recoveries of subcellular 'markers' from post-mitochondrial supernatant treated with concanavalin A-agarose are shown in table 1. The recoveries of total protein, RNA and glucose-6-phosphatase activity were essentially complete; those of cholesterol and 5′-nucleotidase activity were strikingly reduced. These data strongly suggest the removal of a subfraction high in both cholesterol and 5′-nucleotidase but low in glucose-6-phosphatase activity. Ouabain-sensitive ATPase measurement proved, in our hands, to be less reliable for routine assay of fractions than 5′-nucleotidase but in a series of 7 experiments the ratios 5′-nucleotidase/glucose-6-phosphatase = 0.62 ± 0.11 (n = 7) and ouabain-sensitive ATPase/5′-nucleotidase = 1.02 ± 0.2 (n = 7) strongly suggested an equal effect of concanavalin A treatment on these two plasma membrane marker activities. The most probable explanation of these findings is that the immobilised lectin binds to, and hence under these conditions removes, fragments of plasma membrane. The substantial fraction of cholesterol removed is consistent with this interpretation.

An attempt was made therefore to label the surface of isolated hepatocytes with a non-permeant reagent – diazotized sulphanilic acid. Liver parenchymal cells were incubated with diazotized [^{14}C]sulphanilic acid (3.5 mM in Hank's medium) for 60 min at 37°C as described above. At the end of this time the 5′-nucleotidase activity had been reduced to approx. 48% of its initial value whereas glucose-6-phosphatase was unaffected (<2% inhibition); in cells broken by gentle homogenization in 1 mM sodium bicarbonate, the 5′-nucleotidase activity was similarly reduced but, under these circumstances, the glucose-6-phosphatase was reduced to less than 20% of initial, or incubated control, value – evidence that the original cells were indeed intact and that diazotized sulphanilic acid could not penetrate the limiting membrane. After incubation, the cells were washed twice with incubation medium to remove unbound ligand and the cell pellet homogenized in the Hepes buffer to prepare post-mitochondrial supernatant as if it had been solid tissue. The supernatant thus obtained was treated with concanavalin A-agarose as described above. The immobilized lectin removed 50 ± 5% (n = 3) of the radioactivity associated with the post-mitochondrial supernatant and, in parallel experiments, 60 ± 6% (n = 3) of the 5′-nucleotidase activity. Recoveries of the glucose-6-phosphatase were essentially complete (>95%). These data reinforce the original suggestion that concanavalin A binds to, and hence may be used to remove, fragments of plasma membrane from microsomal preparations.

Attempts to elute the material bound to the concanavalin A-agarose with α-methyl mannoside met with only limited success. The gel was washed on the sinter with 3 vols of 0.25 M α-methyl mannoside and the resulting eluent analysed (table 2). Increasing the concentration of α-methyl mannoside to 0.5 M did not increase the recovery of 'lost' markers.

Although fractions with high cholesterol content and 5′-nucleotidase activity compared with glucose-6-phosphatase were obtained the total recovery of these markers was disappointingly low (approx. 25% of the 5′-nucleotidase activity and 20% of the cholesterol removed by lectin treatment). Of the missing 5′-nucleotidase, approx. 70% could be detected in the α-methyl mannoside-treated beads (accounting for a total recovery of 78%).

In summary, we have demonstrated the concanavalin A-mediated removal of a subfraction of the microsomal fraction of rat liver that is associated with 5′-nucleotidase, ouabain-sensitive

Table 2

Characteristics of material eluted from concanavalin A-agarose and by subsequent treatment with α-methyl mannoside

	5′-Nucleotidase / G-6-Pase	$Chol^{free}$ / PL	$Chol^{total}$ / PL
Unfractionated PMS	1.00	1.00	1.00
Concanavalin A 'filtrate'	0.41 ± 0.05(4)	0.79 ± 0.04(8)	0.69 ± 0.02(6)
Activity eluted from treated gel by α-methyl mannoside	9.4 ± 2.9 (3)	3.55 ± 0.6 (2)	–
Residual activity in concanavalin A-agarose gel	28.6 ± 1.8 (3)	4.9 ± 0.3 (2)	–

Values given are ratios of percent recoveries in the fractions indicated ± SE (number of separate experiments in parentheses); PMS, post-mitochondrial supernatant; G-6-Pase, glucose-6-phosphatase; Chol, cholesterol; PL, phospholipid

ATPase and a relatively high level of cholesterol. Glucose-6-phosphatase, on the other hand, is absent, or present at very low levels, in this subfraction. We believe that these findings identify the subfraction removed as plasma membrane. The removal of plasma membrane from microsomal prepartions allows more accurate determination of the characteristics and activities of the residual membrane, deriving principally from the endoplasmic reticulum. The present data, for example, indicate that a substantial proportion of microsomal cholesterol is associated with the 'contaminating' plasma membrane fragments. Affinity methods may also be of more general use in rapid and gentle methods of subcellular fractionation. A previous report [6] that different lectins bind specifically to different cell surfaces suggests a method for the subfractionation of plasma membrane from different cell types in complex, solid tissues.

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