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REMOVAL OF PERCOLL FROM MICROSOMAL VESICLES BY GEL FILTRATION ON SEPHACRYL S-1000 SUPERFINE

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A microsomal vesicle fraction was prepared from rat liver homogenate by centrifugation in gradients of Percoll®. The microsomes were subjected to gel filtration on Sephacryl® S-1000 Superfine, which resolved the microsomes from Percoll. The elution pattern of the microsomal marker enzyme NADPH-cytochrome *c* reductase showed that the main part of the enzyme was present in a peak at K_{av} about 0.1, while Percoll eluted in a broad peak at K_{av} about 0.7. The total yield of eluted enzyme activity was 85%. The gel filtration had to be carried out in the presence of 10 mM Tris or NaCl. At lower ionic strength or in 0.25 M sucrose alone, anomalous behaviour of the Percoll particles and microsomes on the gel was observed. Electron microscopy of samples from the void volume fraction of the Sephacryl S-1000 Superfine column showed an almost complete removal of Percoll from the microsomes. Furthermore, the vesicle preparation was essentially free of membrane fragments.

Introduction

Percoll® is a recently introduced density gradient centrifugation medium which consists of polyvinylpyrrolidone-coated silica particles [1]. Its physicochemical properties have been described [2–4]. Percoll has low viscosity and is easily rendered isotonic, properties which make it very useful for separation of cells and subcellular particles (for a review see Ref. 5). Its uses in subcellular fractionation include the separation of mitochondria [6,7], lysosomes [8], peroxisomes [9], chromaffin granules [10], rat renal cortex membranes [11] and plasma membranes from rat liver cells [12], platelets [13], epithelial cells [14] and fat cells [15].

One disadvantage with the use of Percoll is its interference in some commonly used methods for protein determination. When using the procedure of Lowry et al. [16] it is necessary to include standards containing known amounts of Percoll to obtain an accurate value of the protein concentration in the sample. The Coomassie blue method [17] has been used with some modifications to obtain reliable values [18]. It has also been shown that the Percoll particles will to some extent bind monovalent cations such as sodium [4]. This finding makes it difficult to carry out assays containing, for example, radioactive sodium in the presence of Percoll. Furthermore, Percoll is seen as a contaminant in electron micrographs due to its electron-dense silica core. It is therefore important to remove the Percoll from the subcellular particles. This can easily be done by centrifugation for large organelles, e.g., mitochondria [6]. Considerable problems do, however, arise with membrane vesicles having a sedimentation rate similar to that of Percoll particles. Centrifugation at $48000 \times g_{av}$ for 30 min has been used to remove Percoll from plasma membrane vesicles obtained from epi-

thelial cells [14]. The Percoll was pelleted from a dilute solution and the membrane vesicles remained above the Percoll cushion. Similar conditions have been used to remove Percoll from secretory vesicles purified from bovine neurohypophyses [19]. The ultracentrifugation procedures have, however, several disadvantages. The centrifugal fields may damage the membrane vesicles and recoveries of protein and enzyme activity are decreased after the centrifugation [14,20]. Further, the vesicles still contain some Percoll particles after the centrifugation. A gel filtration procedure has been used to remove Percoll from secretory vesicles [21]. The gel filtration was carried out on Bio-Gel® A-150; however, agarose gels generally exhibit low flow rates and long separation times are required. This paper describes a rapid and gentle technique to remove Percoll from membrane vesicles based on a gel filtration procedure using the new gel matrix Sephacryl S-1000 Superfine.

Materials and Methods

Livers from male Sprague-Dawley rats were homogenized in 0.25 M sucrose. A post-nuclear homogenate was prepared by centrifugation of the liver suspension at $600 \times g_{av}$ for 10 min.

A modified procedure according to Ravin et al. [22] was used to label Percoll (Pharmacia Fine Chemicals). To 3 ml Percoll was added 0.2 ml of 20% Na_2CO_3 (solution A). Separately, 0.1 mg NaI (10 µl, 10 mg/ml) containing 0.2 mCi (7.4 MBq) carrier-free $Na^{125}I$ was mixed with 1 ml 1% $NaNO_2$ and 1 ml 0.1 M HCl (solution B). Iodination was carried out by mixing solution A with solution B immediately. The mixture was dialysed against distilled water for 4 d with a change of water twice a day. About 7 ml ^{125}I-labelled Percoll was obtained with a specific activity of $3.6 \cdot 10^6$ cpm/ml. The amount of free ^{125}I was less than 1%, determined from the outer solution from the last dialysis.

Stock isotonic Percoll (SIP) was prepared by dissolving 8.56 g sucrose in 90 ml of undiluted Percoll in a measuring flask and diluting to 100 ml with water. SIP was diluted to required concentrations with 0.25 M sucrose.

In order to obtain a microsomal fraction, 60 ml 50% SIP was mixed with 10 ml post-nuclear homogenate. In some experiments 1 ml of ^{125}I-labelled Percoll was added. The mixture was centrifuged at $77000 \times g_{av}$ in an MSE centrifuge for 1.5 h at 20°C. The gradient formed was monitored by use of Density Marker Beads (Pharmacia Fine Chemicals) in a control tube in which the post nuclear homogenate had been substituted by 0.25 M sucrose. The band containing the microsomal activity was collected.

NADPH-cytochrome *c* reductase (EC 1.6.2.4) was used as a marker for microsomes and was determined according to Sottocasa [23]. The presence of Percoll did not affect the enzyme activities (data not shown). Protein was determined according to Bradford [17]. No correction for Percoll was made.

Gel filtration was performed in K 16/40 columns (Pharmacia Fine Chemicals) containing 60 ml Sephacryl S-1000 Superfine (Pharmacia Fine Chemicals). These columns were developed in 0.25 M sucrose containing 10 mM Tris (pH 7.4) with a flow rate of 51–53 $ml \cdot h^{-1}$. In some experiments the Tris buffer was omitted. Microsomal vesicles (5 ml) containing 94000–137000 cpm ^{125}I-labelled Percoll were applied on the column. The sample was made 10 mM in Tris buffer by the addition of 50 µl 1 M Tris (pH 7.4) prior to application. The absorbance at 280 nm was followed with a UV-2 monitor (Pharmacia Fine Chemicals). The radioactivity, NADPH-cytochrome *c* reductase activity and protein were determined in each fraction. The total elution time was about 60 min. The elution position of the microsomes and Percoll was expressed as K_{av}, calculated according to Laurent and Killander [24]. For gel filtration studies of Percoll alone the medium centrifuged to remove aggregates and a fraction in the density range of 1.05–1.08 g/ml was collected. Samples of this fraction (2 ml) were applied to a Sephacryl S-1000 Superfine column equilibrated in 0.25 M sucrose containing different concentrations of Tris or NaCl.

Samples from the microsomal peak were analyzed by electron microscopy. A negative contrast technique was used with 2% phosphotungstic acid as a contrasting agent. The specimens were examined in a Philips EM 300 electron microscope using an operating voltage of 60 kV.

Results and Discussion

Gel filtration of microsomes containing ^{125}I-labelled Percoll was carried out on Sephacryl S-1000 Superfine in the presence and absence of 10 mM Tris (pH 7.4) (Fig. 1a and 1b, respectively). These experiments show that the addition of 10 mM Tris to the microsomes is necessary for proper gel filtration behaviour. Experiments with Percoll alone have shown that the optimal concentration of Tris is 10 mM. The elution pattern of Percoll in 10 mM NaCl was the same as in 10 mM Tris. These data show that the presence of ions is necessary for gel filtration of Percoll but that the type of ion is less important. The anomalous behaviour of Percoll particles in the experiment without Tris buffer (Fig. 1b) could probably be explained by larger effective particle volumes at low ionic strength. It has been shown [2,3] that the Percoll particles have a much increased hydrodynamic volume in the absence of salt. An alternative explanation to the anomalous gel filtration behaviour of Percoll as shown in Fig. 1b may be an ionic exclusion of the slightly negatively charged Percoll particles from the gel due to the presence of a small amount of negatively charged groups on the gel matrix. The total yield of radioactivity from the gel was about 85%.

The elution pattern of microsomes on Sephacryl S-1000 Superfine was followed by analysing the microsomal marker enzyme NADPH-cytochrome *c* reductase. The result is given in Fig. 2. The main part of the enzyme (60% of total) was present in a peak at a K_{av} value of about 0.1, while Percoll eluted in a broad peak at a K_{av} value of about 0.7. Enzyme activity can, however, be found in all fractions after the void volume. This is probably due to elution of very small vesicles and membrane fragments. The total yield of marker enzyme was 85% of the activity applied on the column.

The fractions around the total volume are slightly red due to contaminating hemoglobin and it is obvious that the bulk of cytoplasmic proteins will elute in the total volume of the column. It has recently been shown [25] that microsomes prepared by gel filtration on Sepharose® 2B give rise to a considerably decreased number of spots in two-dimensional electrophoresis, compared with microsomes prepared by differential centrifugation, due to the removal of cytoplasmic proteins. Therefore a gel filtration procedure included in the preparation of microsomes will be an advantage in the study of membrane-bound proteins related to the endoplasmic reticulum.

Electron microscopy was used to analyse the microsomal fraction for contaminating Percoll

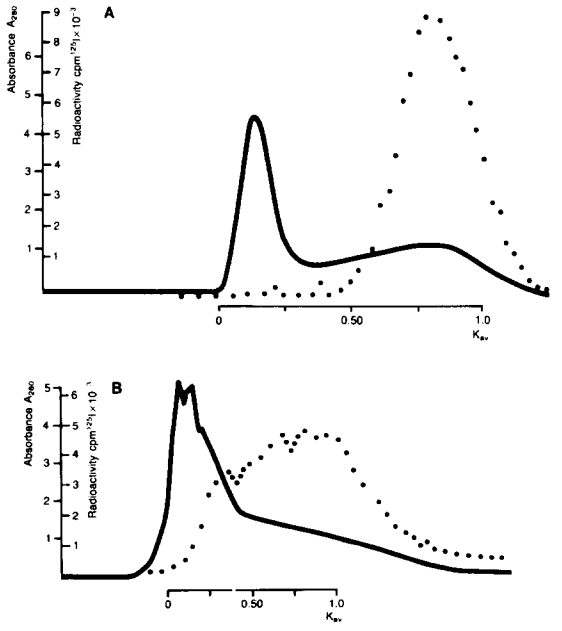

Fig. 1. Gel filtration of microsomes obtained from gradients of Percoll containing ^{125}I-labelled Percoll on (a) Sephacryl S-1000 Superfine in 0.25 M sucrose containing 10 mM Tris (pH 7.4); (b) Sephacryl S-1000 Superfine in 0.25 M sucrose. ————, A_{280}; · · · · · ·, radioactivity.

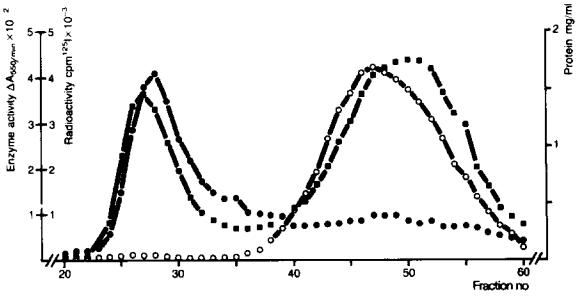

Fig. 2. Distribution of the microsomal marker enzyme NADPH cytochrome *c* reductase, radioactivity and protein after gel filtration of microsomes on Sephacryl S-1000 Superfine. Fraction volume was 1 ml. ●———●, Enzyme activity; ○———○, radioactivity; ■———■, protein.

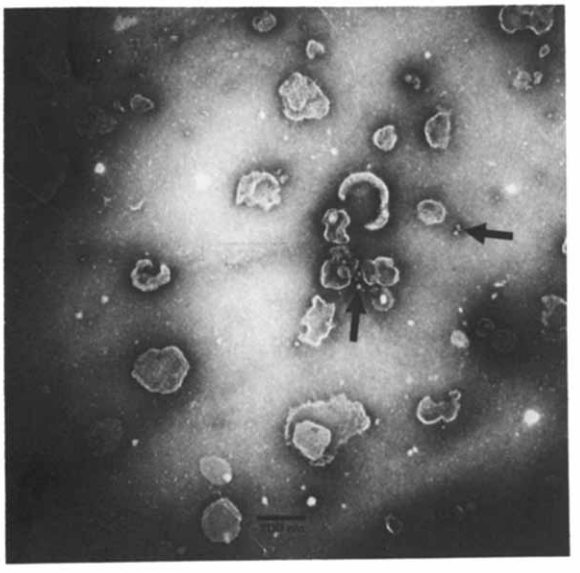

Fig. 3. Electron micrograph of sample from the microsomal peak (fraction 28). Arrows indicate Percoll particles left after gel filtration.

particles after gel filtration. As seen in Fig. 3 the microsomal fraction is almost free from Percoll particles. The fraction of Percoll particles left after gel filtration is less than 0.5% compared to the initial sample. The vesicles are undamaged and essentially free from membrane fragments.

In conclusion, this method makes it possible to remove Percoll from vesicles more gently than with the currently used centrifugation techniques. The yield of vesicles is similar or somewhat better.

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