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The isolation of endosome-derived vesicles from rat hepatocytes

J. Paul LUZIO* and Keith K. STANLEY†
**Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K., and †European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 6900 Heidelberg, Federal Republic of Germany*

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Intracellular 5'-nucleotidase involved in membrane circulation in rat hepatocytes is latent, and is protected from inhibition when whole cells are incubated with inhibiting antiserum at 2°C [Stanley, Edwards & Luzio (1980) *Biochem. J.* **186**, 59–69]. These two criteria were used to identify intracellular membrane vesicles containing 5'-nucleotidase on Ficoll density gradients. A sharply defined turbid band containing intracellular 5'-nucleotidase isolated on density gradients was further fractionated by immunoadsorption of plasma-membrane fragments derived from the cell surface of surface-inhibited cells on to an anti-(immunoglobulin G) immunoadsorbent. The resulting non-adsorbed membrane fraction consisted of vesicles of uniform size (approx. 65nm diam.), but was not identifiable as any known organelle. This fraction could account for approx. 5% of the total cell 5'-nucleotidase activity, and the enzyme activity measured was 55% latent. The fraction had a restricted polypeptide composition but similar phospholipid composition compared with plasma membrane. We suggest that the vesicles observed in this fraction were derived from the endocytic pathway.

The circulation through the cell of endocytosed plasma-membrane components is supported by much evidence, and there is considerable information about the routes within the cell taken by endocytic vesicles containing specific membrane components or cell-surface-bound ligands (Silverstein *et al.*, 1977; Thilo & Vogel, 1980; Mellman *et al.*, 1980; Steer & Ashwell, 1980; Holtzmann, 1981; Pearse & Bretscher, 1981; Palade, 1982; Goldstein *et al.*, 1982). Such routes include the re-cycling of endocytosed membrane components back to the cell surface, which may involve elements of the Golgi apparatus or lysosomes (Schneider *et al.*, 1979; Ottosen *et al.*, 1980).

It is now generally accepted that clathrin-coated pits are the site of membrane internalization during receptor-mediated endocytosis (Goldstein *et al.*, 1979; Pearse & Bretscher, 1981), but the nature of intracellular vesicles formed after internalization of cell-surface components remains the object of speculation. The formation of uncoated endocytic vesicles has been established, and these have been given different names, including 'receptosomes' (Pastan & Willingham, 1981), 'PIC' (peripheral intermediate compartment; Hubbard, 1982), 'CURL' (the compartment of uncoupling of receptor and ligand; Geuze *et al.*, 1983), and, more broadly, 'endosomes' (see, e.g., Mellman, 1982). There is now extensive electron-microscopic evidence for such an endosome compartment within the cell, though less progress has been made with regard to its isolation (Debanne *et al.*, 1982; Merion & Sly, 1983; Quintart *et al.*, 1983).

One well-defined cell-surface component often used as a plasma-membrane marker (Evans, 1978) that has been shown to circulate in a variety of cells is the ectoenzyme 5'-nucleotidase (EC 3.1.3.5; Stanley *et al.*, 1980; Widnell *et al.*, 1982). In rat hepatocytes approx. 50% of the enzyme activity is latent, i.e. available to substrate only when cells are broken. It has been shown that, whereas both cell-surface and latent activities are inhibited by incubating intact cells with inhibiting antiserum at 37°C, only the cell-surface activity is inhibited when the incubation is performed at 2°C. This latter observation has allowed the demonstration of circulation of the enzyme, since surface-inhibited cells incubated at 37°C show the reappearance of 5'-nucleotidase at the cell surface (Stanley *et al.*,

Abbreviation used: IgG, immunoglobulin G; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid; SDS, sodium dodecyl sulphate; Pipes, 1,4-piperazinediethanesulphonic acid.

1980). Similar experiments have confirmed these observations in fibroblasts, where in addition the circulation of surface bound IgG from the anti-(5′-nucleotidase) serum has been demonstrated (Widnell *et al.*, 1982).

The intracellular location of the 50% of total cell 5′-nucleotidase activity that is latent in hepatocytes is unknown, though both biochemical and cytochemical evidence has been obtained for the presence of some enzyme in the Golgi, lysosomes and microsomal fractions (Widnell & Little, 1977). On the basis of previous experiments with isolated hepatocytes, we have suggested that intracellular enzyme may be found with its active site on the inside of intracellular vesicles (Stanley *et al.*, 1980). Such vesicles when isolated would have latent 5′-nucleotidase activity, and in addition their enzyme activity should be inhibited if intact cells are incubated with anti-(5′-nucleotidase) serum at 37°C before cell disruption, but not if the cells are incubated with antiserum at 2°C. In the present study these criteria for intracellular membrane-bound 5′-nucleotidase are used to demonstrate the endocytic origin of vesicles in subcellular fractions prepared from hepatocyte homogenates by using density-gradient centrifugation and immunoaffinity techniques.

Experimental

Cells

Hepatocytes were isolated by collagenase perfusion of the liver *in situ* by the method of Berry & Friend (1969) as modified by Westwood *et al.* (1979). They were resuspended in Hanks buffer as previously described (Stanley *et al.*, 1980).

Antisera and immunoadsorbents

Rabbit antiserum to immunoaffinity-purified 5′-nucleotidase (Bailyes *et al.*, 1982) was prepared by using the injection schedule previously described (Stanley *et al.*, 1980). It was treated at 56°C for 30min before use to inactivate complement. Sheep anti-(rabbit IgG) immunoadsorbent was prepared by coupling IgG from appropriate antiserum as previously described (Westwood *et al.*, 1979).

Incubation of cells with antiserum

Portions (15ml) of isolated hepatocytes (approx. 20mg dry wt./ml) were cooled at 2°C for 1h and then incubated with 150μl of anti-(5′-nucleotidase) serum for 1h at 2°C to inhibit cell-surface 5′-nucleotidase activity. For inhibition of the intracellular pool of enzyme, the antiserum was added to the cells at 37°C and the cells were incubated for various times up to 1h. Surface-inhibited cells subsequently used in cell-fractionation experiments were washed with 4 × 10ml of cold Hanks buffer (with centrifugation at 800*g* for 2min at 2°C between washes) before suspension in homogenization medium to remove excess antiserum in solution.

Homogenization and subcellular fractionation

Cells were resuspended in buffered sucrose at 2°C before homogenization. The buffered sucrose solution used throughout for both cells and subcellular fractions was 0.25M-sucrose/1mM-$MgCl_2$/10mM-Tes adjusted to pH7.4 with NaOH. Samples (15ml, approx. 20mg dry wt. of cells/ml) were homogenized with the Polytron for 60s at setting 8, and centrifuged at 10000*g* for 10min at 2°C. The Polytron setting used resulted in approx. 50% of total homogenate 5′-nucleotidase being found in the 10000*g* supernatant. Samples (5ml) of the 10000*g* supernatant were loaded on 30ml pre-formed Ficoll gradients consisting of 1–15% (w/v) Ficoll prepared in 0.25M-sucrose/1mM-EDTA/10mM-Tes adjusted to pH7.4 with HCl. The gradients were centrifuged at 100000*g* for 16h at 2°C in a swing-out rotor, then pumped out and collected as 1ml fractions. For preparation of the small-vesicle fraction by treatment with immunoadsorbent, Ficoll-gradient fractions from homogenates of surface-inhibited cells were diluted 1:5 with buffered sucrose (to a total volume of 15–50ml). The diluted fractions were then incubated with 15mg of sheep anti-(rabbit IgG) immunoadsorbent for 2h at 4°C. After this incubation immunoadsorbent was removed by centrifugation at 1700*g* for 10min. For electron microscopy, the vesicle fraction was concentrated by running 5ml portions of this supernatant into 1–15% (w/v) Ficoll gradients as above. For SDS/polyacrylamide-gel-electrophoretic analysis, phospholipid analysis and marker-enzyme content single Ficoll-gradient-peak fractions (rather than the pooled fractions in 2.8–5.3%-Ficoll range) from several gradients were pooled and diluted with buffered sucrose before immunoadsorbent treatment. After this treatment the vesicle fraction was concentrated by centrifugation at 100000*g* for 1.5h and resuspension of the pellet in 1–2ml of buffered 0.25M-sucrose.

Chemical and enzyme assays

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, after precipitation of protein with 10% (w/v) trichloroacetic acid and re-dissolving it in 1M-NaOH. Phosphate was determined by the method of Itaya & Ui (1966). Phosphorus was assumed to account for 4% by weight of phospholipids (Kremmer *et al.*, 1976). Phospholipid analysis was performed by extracting membranes with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957), spotting the extracts on silica-gel t.l.c. plates, developing these in chloroform/methanol/acetic

acid/water (45:25:10:1, by vol.), identifying spots with iodine vapour, and measuring phosphate content after extraction with chloroform/methanol (2:1, v/v). The chromatography system used in phospholipid analysis clearly separated the major membrane phospholipids (sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine). Rabbit IgG was determined by immunoradiometric assay (Beck & Hales, 1975).

SDS/polyacrylamide-gel electrophoresis was by the method of Laemmli (1970) conducted on slab gels containing 10% (w/v) acrylamide with 0.27% (w/v) *NN'*-methylenebisacrylamide. Samples were reduced and alkylated (Lane, 1978) before application to the gels. Gels were stained for protein by the method of Fairbanks *et al.* (1971).

5'-Nucleotidase activity was assayed by conversion of [^{3}H]AMP into [^{3}H]adenosine (Avruch & Wallach, 1971) as described previously (Newby *et al.*, 1975). 5'-Nucleotidase assay medium contained 0.25 M-sucrose, 2 mM-$MgSO_4$, 20 mM-sodium β-glycerophosphate, 1.5 mM-adenosine, 0.2 mM-AMP, 50 mM-Tris, buffered to pH 7.4 with HCl. Activities were determined with and without the presence of 1% Triton X-100, and latent activity was defined as the difference between these. Cytochrome oxidase (EC 1.9.3.1) was assayed by the method of Cooperstein & Lazarow (1951), glucose 6-phosphatase (EC 3.1.3.9) as described by Stanley *et al.* (1980), β-*N*-acetylglucosaminidase (EC 3.2.1.30) by the method of Barrett & Heath (1977), but with 0.5 mM substrate plus 0.1% Triton X-100 at pH 5.0 for 3 min at 37°C, and galactosyltransferase (EC 2.4.1.38) by the method of Bretz *et al.* (1980).

Electron microscopy

Ficoll-gradient fractions were diluted 1:5 with phosphate-buffered saline (0.15 M-NaCl, 2 mM-NaH_2PO_4, 16 mM-Na_2HPO_4) and centrifuged at 120000 *g* for 1 h at 4°C. Pellets were fixed in 1% (w/v) OsO_4 (in phosphate-buffered saline) for 30 min at 20°C and washed three times with 10% (w/v) sucrose/10 mM-Pipes at pH 7.0. They were then dehydrated, embedded, sectioned and examined as previously described (Howell & Palade, 1982).

Results

5'-Nucleotidase activity found in the post-10000 *g* supernatant of rat hepatocyte homogenates (Table 1) was fractionated by centrifugation into Ficoll density gradients, producing the pattern of enzyme activity shown in Fig. 1. There was a peak of enzyme activity coincident with a visible band at 4% (w/v) Ficoll (Fig. 2), and one-third of the enzyme activity in this region of the gradient was latent (Table 1). The subcellular origin of the latent enzyme activity found throughout the gradient [16 ± 1% (mean ± S.E.M.; $n = 6$) of original cell activity] was investigated by studying its distribution on Ficoll gradients of post-10000 *g* fractions from surface-inhibited cells. In such gradients (Fig. 3) the latent enzyme activity represented a much higher proportion of total activity than in the control cell gradients (Fig. 1), consistent with it originating from an intracellular endosomal pool of enzyme. The recovery of latent enzyme on the gradients from surface inhibited cells was 13 ± 1% (6) of original control cell activity, i.e. 81% of the latent activity on control cell gradients (Fig. 1).

The data from surface-inhibited cells suggested that the latent 5'-nucleotidase activity derived from the intracellular pool of enzyme was mixed with enzyme from the cell surface throughout the Ficoll gradient, since total enzyme activity in the gradient fractions was inhibited more than the intracellular activity in surface-inhibited cells. This conclusion was supported by comparison of the inhibition of the major 5'-nucleotidase peak on Ficoll gradients (2.8–5.3% Ficoll) with the degree of inhibition of the cell surface and intracellular pools after whole cells were treated with antibody for various times at 37°C

Table 1. *5'-Nucleotidase activity in hepatocyte fractions*

Cells and fractions were isolated and assayed for 5'-nucleotidase activity as described in the text. Latent activity was measured as the difference between activity in iso-osmotic medium and the same medium plus 1% Triton X-100. Data are expressed relative to total 5'-nucleotidase in control cells (plus 1% Triton X-100), as means ± S.E.M. for six experiments. Control cell activity was 23.1 ± 1.4 units/g dry wt. (38 ± 2 munits/10^6 cells). Surface-inhibited cells were cells that had been incubated with anti-(rat 5'-nucleotidase) serum at 2°C.

	Control cells		Surface-inhibited cells	
	Total	Latent	Total	Latent
Cells	100	58 ± 3	53 ± 2	47 ± 2
10000 *g* supernatant	46 ± 2	18 ± 1	26 ± 1	13 ± 2
Ficoll peak (2.8–5.3% Ficoll)	18 ± 4	6 ± 1	10 ± 1	5 ± 1
Post-immunoadsorbent small-vesicle fraction	—	—	5.3 ± 0.5	2.7 ± 0.3

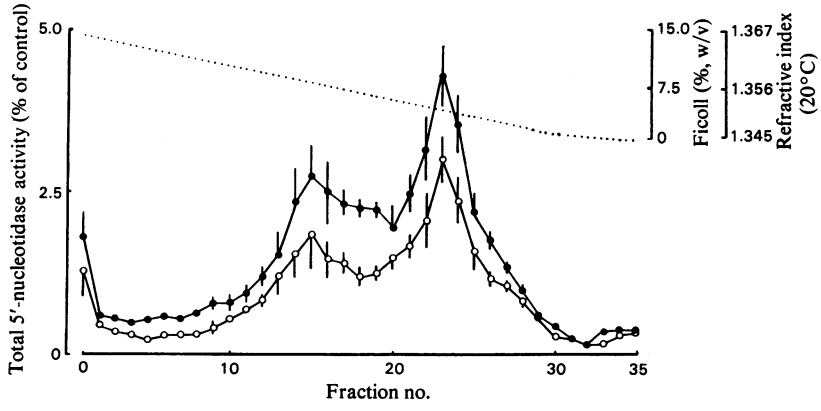

Fig. 1. *Distribution of hepatocyte 5'-nucleotidase in a 1–15% Ficoll gradient*
Portions (5ml) of the 10000*g* supernatants from homogenates of rat hepatocytes were loaded on to 30ml 1–15% Ficoll gradients (••••) and centrifuged at 100000*g* for 16h at 4°C. Gradient fractions (1ml) were collected, and 5'-nucleotidase activity was assayed in the presence (●) or absence (○) of 1% Triton X-100. Each point on the graph represents the mean ± S.E.M. for results from six separate preparations of hepatocytes. Recovery of 5'-nucleotidase (+1% Triton X-100) from the gradient was 90 ± 3% of loaded activity.

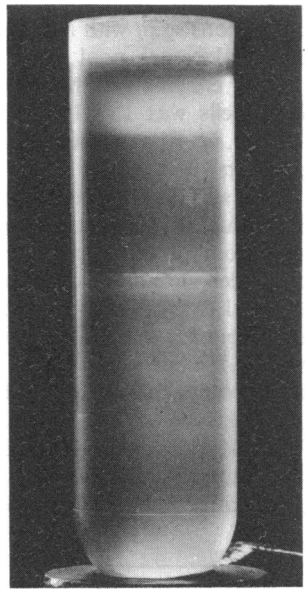

Fig. 2. *Photograph of Ficoll gradient after loading 10000g supernatant from hepatocyte homogenate and centrifugation at 10000g for 16h at 4°C*
A white band is visible, coincident with the peak of 5'-nucleotidase activity.

(Fig. 4). If the peak of 5'-nucleotidase activity (2.8–5.3% Ficoll) derived only from the cell surface, it should be inhibited to the same extent as the cell surface; if it derived only from the intracellular pool, inhibition should equal that of the latent cell enzyme. In all cases, however, the Ficoll-gradient-peak 5'-nucleotidase activity was less inhibited than the cell-surface activity and more inhibited than that of the intracellular pool (Fig. 4), suggesting that it was derived from both. This conclusion probably applies to all the 5'-nucleotidase activity seen on the Ficoll gradients, since similar latency and inhibition of 5'-nucleotidase in response to extracellular antibody was observed in all fractions.

The distribution of marker enzymes on the Ficoll gradient was measured after centrifuging the post-10000*g* supernatant from control cells into the gradient as described for Fig. 1. The peak of 5'-nucleotidase activity was well separated from cytochrome oxidase and glucose 6-phosphatase, which pelleted to the bottom of the centrifuge tube (Fig. 5). In the Ficoll gradients there was considerable overlap of β-*N*-acetylglucosaminidase, galactosyltransferase and 5'-nucleotidase activities (Fig. 5), though the peaks of activity were separated, suggesting that the 5'-nucleotidase, including latent enzyme, was not simply derived from Golgi apparatus or lysosomes. Much of the β-*N*-acetylglucosaminidase activity found in the 5'-nucleotidase-peak region of the Ficoll gradient appeared to be free enzyme, since, when fractions from this region of the gradient were diluted with buffered 0.25M-sucrose and centrifuged for 1h at 200000*g*, 85% of 5'-nucleotidase and 70% of galactosyltransferase were pelleted, but only 12% of β-*N*-acetylglucosaminidase. When IgG distribution was measured on the Ficoll gradient after loading and centrifuging the post-10000*g* supernatant from cells

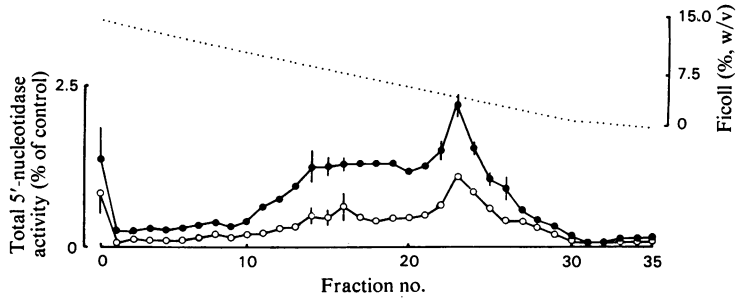

Fig. 3. *Distribution of 5'-nucleotidase from surface-inhibited hepatocytes in a 1–15% Ficoll gradient*
Cell-surface 5'-nucleotidase activity was inhibited by incubating hepatocytes with anti-(5'-nucleotidase) serum for 1 h at 2°C. Cells were washed, homogenized and centrifuged at 10000 *g* for 10 min. Samples (5 ml) of the 10000 *g* supernatant were loaded on to 30 ml 1–15% Ficoll gradients (••••) and centrifuged at 100000 *g* for 16 h at 4°C. Gradient fractions (1 ml) were collected and 5'-nucleotidase activity was assayed in the presence (●) or absence (○) of 1% Triton X-100. Each point on the graph represents the mean ± S.E.M. for results from six separate experiments. Control cells, from the same hepatocyte preparations, without inhibition of surface 5'-nucleotidase were homogenized and treated identically, the data being shown in Fig. 1.

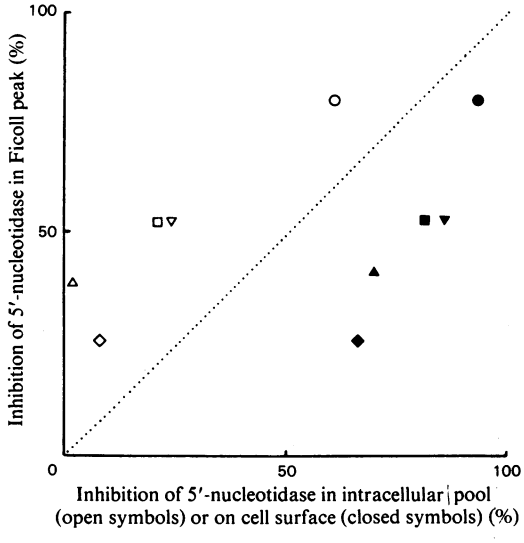

Fig. 4. *Comparison of the inhibition of 5'-nucleotidase in peak fractions from the 1–15% Ficoll gradient with the inhibition of cell-surface or intracellular 5'-nucleotidase activity*
Hepatocytes were incubated with rabbit anti-(rat 5'-nucleotidase) serum at 37°C for various times to give different amounts of inhibition of both cell-surface and intracellular 5'-nucleotidase pools (Stanley *et al.*, 1980). Cells were then washed, homogenized and centrifuged at 10000 *g* for 10 min. Samples (5 ml) of the 10000 *g* supernatant were loaded on to 30 ml 1–15% Ficoll gradients and centrifuged at 100000 *g* for 16 h at 4°C. Gradient fractions (1 ml) were collected and 5'-nucleotidase activity was assayed in the presence of 1% Triton X-100. The total activity in the peak of 5'-nucleotidase (2.8–5.3% Ficoll) was calculated, and percentage inhibition relative to the peak activity of control (non-inhibited) cells plotted against percentage inhibition of the cell-surface (closed symbols) or internal pool (open symbols) of 5'-nucleotidase in the intact inhibited hepatocytes. Different symbols represent measurements from different experiments. The dotted line shows the inhibition of Ficoll-gradient-peak 5'-nucleotidase activity expected if it were directly proportional to either cell-surface or internal-pool inhibition.

in which surface 5'-nucleotidase activity was antibody-inhibited, the IgG profile closely followed the residual 5'-nucleotidase activity (results not shown). Since this IgG represents cell-surface-bound antibody, these results are again consistent with plasma membrane being distributed through the gradient in a similar way to 5'-nucleotidase from the intracellular pool.

Transmission electron microscopy of membrane in the visible band on the Ficoll gradient revealed the presence of membrane vesicles of various sizes (Fig. 6*a*). The vesicles seen had the same appearance when prepared from cells in which surface 5'-nucleotidase was antibody inhibited (results not shown). From the experiments described above it was apparent that some of these vesicles must have derived from the cell surface and would be expected to contain surface-bound anti-(5'-nucleotidase) antibodies. These vesicles might be removed by incubation with an anti-IgG immunoadsorbent, allowing purification of intracellular-derived vesicles. The pooled data from six experiments showed that

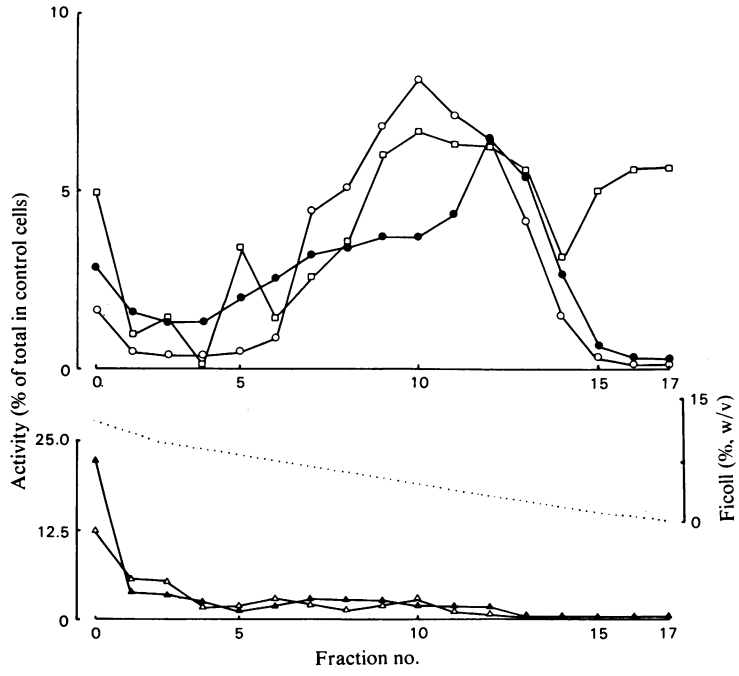

Fig. 5. *Recovery of marker enzymes in a 1–15% Ficoll gradient*
Samples (5 ml) of the 10000 ***g*** supernatants from homogenates of rat hepatocytes were loaded on to 30 ml 1–15% Ficoll gradients (••••) and centrifuged at 100000 ***g*** for 16 h at 4°C. Gradient fractions (2 ml) were collected, and the enzymes 5′-nucleotidase (+1% Triton X-100; ●), cytochrome oxidase (▲), glucose 6-phosphatase (△), galactosyltransferase (□) and *β-N*-acetylglucosaminidase (○) were assayed. Data from a single representative gradient are shown. In this experiment, total recoveries were: cytochrome oxidase, 64%; 5′-nucleotidase, 67%; other enzymes, >90%.

Table 2. *Phospholipid composition of small-vesicle fraction compared with plasma membrane*
Values are expressed as mol % (means ± S.E.M.) of total phospholipid phosphate loaded on t.l.c. plates in three separate experiments. Small-vesicle fractions were prepared by Ficoll-density-gradient centrifugation and immunoadsorbent treatment. Liver plasma-membrane fractions were prepared by the method of Pilkis *et al.* (1974). The range of values reported for liver plasma-membrane phospholipid composition was taken from Kremmer *et al.* (1976), and that for liver Golgi membrane from Zambrano *et al.* (1975) and Howell & Palade (1982).

Phospholipids	Small-vesicle fraction	Liver plasma membrane	Range of values reported for liver plasma membrane	Range of values reported for liver Golgi membrane
Sphingomyelin	15.0 ± 5.7	11.8 ± 3.4	10.7–24.4	7.6–8.3
Phosphatidylcholine	37.5 ± 7.1	26.8 ± 3.8	30.0–46.7	49.6–58.9
Phosphatidylinositol	4.0 ± 1.9	13.0 ± 6.0	4.2–10.3	4.4–12.2
Phosphatidylserine	12.3 ± 1.3	13.7 ± 1.3	3.5–9.0	3.1–5.6
Phosphatidylethanolamine	26.8 ± 8.9	21.5 ± 0.5	14.0–24.7	19.6–20.9

85 ± 5% of the membrane-bound IgG was removed when the Ficoll-peak fraction from surface-inhibited cells was incubated with anti-IgG immunoadsorbent (Table 1); 45 ± 5% of the 5′-nucleotidase in the Ficoll peak fraction was also removed in this incubation. The remaining membrane, which was interpreted as being of intracellular origin, showed 55% latency of 5′-nucleotidase activity, banded in the expected region (2.8–5.3% Ficoll) when centrifuged into a Ficoll gradient, and on transmission

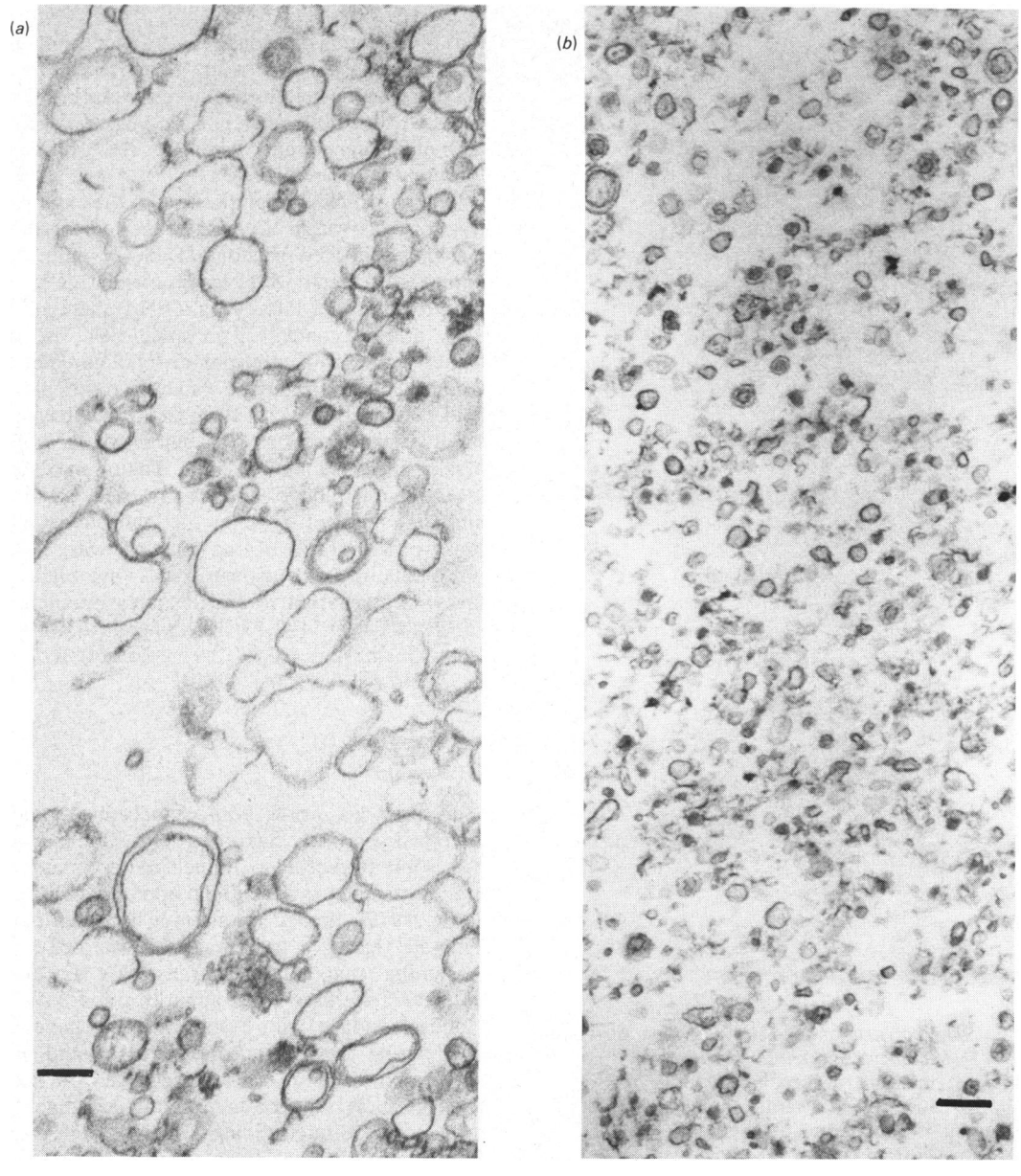

Fig. 6. *Electron micrographs of Ficoll-gradient fractions*

(*a*) Membrane vesicles in the Ficoll-peak fraction (4% Ficoll) from control hepatocytes. (*b*) Membrane vesicles in the Ficoll-peak fraction (4% Ficoll) after inhibition of cell-surface 5′-nucleotidase, Ficoll-density-gradient centrifugation, immunoadsorbent treatment and re-centrifugation on the Ficoll density gradient. Bar represents 200nm in (*a*) and (*b*).

electron microscopy consisted of a uniform population of small vesicles, approx. 65nm diam. (Fig. 6*b*).

In three experiments where the small-vesicle fraction was prepared by immunoadsorbent treatment of single Ficoll-peak fractions the recovery of total cell activity was 5′-nucleotidase $2.8 \pm 0.2\%$, galactosyltransferase $1.6 \pm 0.4\%$ and β-*N*-acetylglucosaminidase $0.3 \pm 0.1\%$. The SDS/polyacrylamide-gel-electrophoresis profile clearly showed contamination of the small-vesicle fraction by protein from the anti-IgG immunoadsorbent (Fig. 7). This

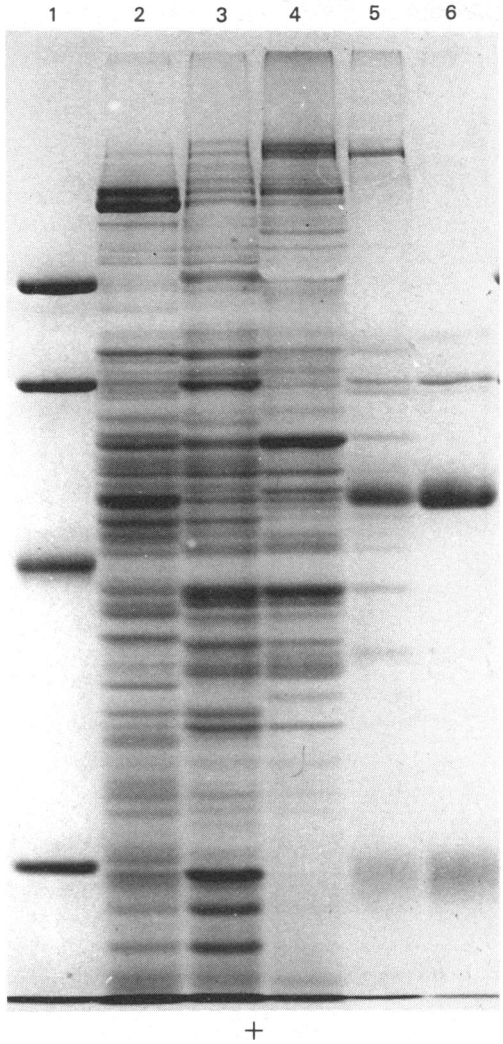

Fig. 7. *SDS/polyacrylamide-gel electrophoresis of hepatocyte subcellular fractions*

Tracks: 1, molecular-weight markers (phosphorylase *b*, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000); 2, 10000 ***g*** pellet from control hepatocytes; 3, 10000 ***g*** supernatant from control hepatocytes; 4, Ficoll-density-gradient peak fraction (4% Ficoll) after inhibition of cell-surface 5′-nucleotidase; 5, small-vesicle fraction from surface-inhibited cells after Ficoll-density-gradient centrifugation and immunoadsorbent treatment; 6, sheep anti-(rabbit IgG) immunoadsorbent. Protein loaded was 10 μg/track.

contamination accounted for 65% of protein loaded when Coomassie Blue-stained gels were scanned. Taking into account this contamination, the specific activity of 5′-nucleotidase in the small-vesicle fraction was 45 ± 7 (7) munits/mg of protein, similar to that of the Ficoll-gradient-peak fraction from control cells [53 ± 6 (15) munits/mg of protein]. On SDS/polyacrylamide-gel electrophoresis, pig brain clathrin (M_r 180000) did not co-migrate with any high-molecular-weight band in the small-vesicle fraction (results not shown).

The phospholipid composition of the small-vesicle fraction was within the range reported for rat liver plasma membrane fractions (Table 2), though when compared directly with plasma membrane prepared by the method of Pilkis *et al.* (1974) there appeared to be a lower content of phosphatidylinositol and a higher content of phosphatidylcholine (Table 2). Compared with reported values for the phospholipid composition of rat liver Golgi membranes, the small-vesicle fraction was enriched in sphingomyelin and phosphatidylserine. Taking into account the contamination of the small-vesicle fraction by immunoadsorbent protein, the phospholipid/membrane-protein ratio of the fraction was 2.0 μg of phospholipid/μg of protein, somewhat higher than the values reported for rat liver plasma-membrane subfractions (0.54–0.77; Kremmer *et al.*, 1976) or Golgi membrane (0.83–0.91; Zambrano *et al.*, 1975; Howell & Palade, 1982).

Discussion

In previous work, both cytochemical and biochemical evidence has been presented suggesting that some intracellular 5′-nucleotidase is associated with lysosomes and the Golgi apparatus (Widnell & Little, 1977), though no attempt has been made to estimate this as a percentage of total cell activity. Assuming that galactosyltransferase is uniquely associated with the Golgi apparatus and β-*N*-acetylglucosaminidase with lysosomes, we have calculated, using our 5′-nucleotidase assay and purified subcellular fractions of each organelle from rat liver, that the Golgi apparatus contains less than 5% of total cell 5′-nucleotidase activity (Hodson & Brenchley, 1976) and lysosomes 3% (Maguire *et al.*, 1983). Clearly, if only 50% of total cell activity is present at the cell surface then approx. 40% must be at previously unidentified intracellular sites. Some confirmation of this deduction has come from immunofluorescent localization of 5′-nucleotidase in cultured rat liver cells by using a monoclonal antibody directed against the enzyme (Stanley *et al.*, 1983). This showed that the intracellular 5′-nucleotidase was more widely distributed than known endoplasmic-reticulum or Golgi antigens.

In the present study a membrane-vesicle fraction of endosomal origin containing approx. 5% of total cell 5′-nucleotidase activity was isolated from rat hepatocytes by using density-gradient and immunoaffinity techniques. By the criteria of 5′-nucleotidase

latency and the response of 5′-nucleotidase activity to the treatment of intact cells with inhibitory antibodies, this membrane fraction represents membrane internalized from the cell surface and on a pathway of circulation. The latency of 5′-nucleotidase in the membrane fraction was over 50%, though the lack of 100% latency may be an artefact of the method of isolation and means of assay, which are known to affect the latency of isolated subcellular organelles (Reijngoud & Tager, 1977; Fleischer, 1981). In the final preparative step using immunoadsorbent treatment, galactosyltransferase was enriched with 5′-nucleotidase, though it seems unlikely in view of the phospholipid differences that the vesicle fraction isolated derives from Golgi membrane. It has been suggested that galactosyltransferase may be associated with the plasma membrane as well as the Golgi apparatus (Shur & Roth, 1975). The membrane fraction isolated, consisting of vesicles of uniform size, may derive from an early stage in the pathway of endocytosis. In addition to the properties described above, it lacks intracellular marker enzymes and has similar phospholipid composition to the plasma membrane, though with less phosphatidylinositol. In contrast, it has a very restricted protein composition compared with plasma membrane.

Immmunoadsorbent techniques have been used previously both in the isolation of plasma membrane from adipocytes (Luzio *et al.*, 1976) and hepatocytes (Westwood *et al.*, 1979) and in the preparation of subcellular organelles (Ito & Palade, 1978). In the present experiments the immunoadsorbent was used simply to remove plasma membrane, leaving what we believe to be vesicles derived from an endocytic pathway. At present we cannot speculate on the relationship of these vesicles with vesicles transporting surface-bound ligands into or across the hepatocyte, or whether such vesicles are associated with the latent 5′-nucleotidase activity found at greater density in the Ficoll gradients. However, the ability to isolate a vesicle population of uniform size should be of value to biochemical studies of endocytosis.

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