Subcellular Distribution and Movement of 5'-Nucleotidase in Rat Cells

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1. Cell-surface 5'-nucleotidase was assayed by incubating whole-cell suspensions with 5' [³H]-AMP in iso-osmotic buffer and measuring [³H]adenosine production. The activity of cell-surface 5'-nucleotidase in hepatocytes, adipocytes and lymphocytes isolated from the rat was 15.0, 0.5 and 0.8 pmol/min per cell at 37°C respectively. 2. Disruption of the cells by vigorous mechanical homogenization or detergent treatment exposed additional 5'-nucleotidase activity, which represented 52%, 25% and 21% of the total activity in the three cell types respectively. This increase in 5'-nucleotidase activity which occurred when the cells were homogenized was due to a second pool of 5'-nucleotidase within the cell, rather than activation of the cell-surface enzyme. 3. In hepatocytes the intracellular 5'-nucleotidase activity was membrane-bound, indistinguishable from cell-surface 5'-nucleotidase in its inhibition by rabbit anti-(rat liver 5'-nucleotidase) serum and its kinetics with AMP, and was located on the extracytoplasmic face of vesicles within the cell. 4. The cell-surface 5'-nucleotidase of rat hepatocytes was rapidly inhibited when rabbit anti-(rat liver 5'-nucleotidase) serum or concanavalin A was added to the medium at 37°C. Incubation with antiserum for 5 min at 37°C inhibited 83 ± 3% of the cell-surface enzyme. 5. Incubation of hepatocytes with exogenous antiserum or concanavalin A for 30 min at 37°C resulted in over 50% inhibition of the intracellular enzyme. This inhibition was not prevented by disruption of the cytoskeleton or by ATP depletion. 6. Incubation of hepatocytes with exogenous antiserum or concanavalin A for up to 2 h at 0°C caused little or no inhibition of the intracellular enzyme, but over 75% inhibition of the cell-surface enzyme. 7. When surface-inhibited hepatocytes were washed and re-suspended in buffer at 37°C, 5'-nucleotidase was observed to redistribute from the intracellular pool to the cell surface.

5'-Nucleotidase (EC 3.1.3.5) is an intrinsic membrane glycoprotein which has been shown in a variety of cell types to be an ectoenzyme, i.e. the active site is located on the outside surface of the cell (DePierre & Karnovsky, 1974; Gurd & Evans, 1974; Trams & Lauter, 1974; Newby *et al.*, 1975). It has been widely used as a plasma-membrane marker enzyme in fractionation studies (DePierre & Karnovsky, 1973; Evans, 1978). However, it remains uncertain whether 5'-nucleotidase is uniquely located in the plasma membrane, since a number of histochemical and biochemical studies have suggested an intracellular location for some of the enzyme.

Histochemical studies on subcellular fractions from liver (Widnell, 1972) and in liver slices (Farquhar *et al.*, 1974) have suggested that in this tissue the enzyme is widely distributed among intracellular organelles. In contrast, Uusitalo & Karnovsky (1977) have suggested from histochemical staining that the 5'-nucleotidase activity in mouse lymphocytes is

exclusively located on the extracytoplasmic surface of the plasma membrane. The localization of 5'-nucleotidase by lead phosphate deposits, however, has many limitations (Widnell, 1972; DePierre & Karnovsky, 1973; El-Aaser *et al.*, 1973; Widnell & Little, 1977). The interpretation of negative results is especially difficult, since they may be explained by lack of penetration of substrate or by inactivation of enzyme with fixatives or lead ions.

Biochemical studies of the extracellular location of 5'-nucleotidase usually employ the latency of the enzyme to exogenous non-penetrating substrate and the ability to inhibit the activity with non-penetrating inhibitors. If the activity of whole cells and homogenates is compared in order to assess the proportion of 5'-nucleotidase on the cell surface, it is necessary to disrupt intracellular organelles. The use of detergents, however, has been avoided because of the possibility of enzyme activation. In guinea-pig polymorphonuclear leucocytes (DePierre & Karnovsky, 1974) and rat adipocytes (Newby *et al.*, 1975) no increase in activity was observed after mechanical homogenization, and it was concluded that most of the activity was on the cell surface. In a variety of

Abbreviation used: SDS, sodium dodecyl sulphate.

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cultured cells (Trams & Lauter, 1974), and in mouse peritoneal macrophages (Edelson & Cohn, 1976), however, evidence was obtained for an intracellular pool of 5'-nucleotidase.

In the present experiments we have investigated both cell-surface 5'-nucleotidase and the extra activity released on homogenization or detergent disruption of rat hepatocytes, adipocytes and lymphocytes. We report the effect of exogenous inhibitors (antiserum and concanavalin A) on hepatocyte cell-surface and intracellular pools of 5'-nucleotidase activity, and we present evidence that a dynamic relationship exists between these two pools of enzyme. A preliminary account of these experiments has appeared (Stanley & Luzio, 1979).

Experimental

Materials

Collagenase (CLS) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and bovine serum albumin (fraction V) from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Concanavalin A, heparin (sodium salt) and α -methyl D-mannoside were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Scintillation-grade Triton X-100 was from BDH Ltd., Poole, Dorset, U.K., and Lubrol 12A9 from Organics Division, ICI, Blackley, Manchester, U.K. All other reagents were the highest grade available. [2-³H]AMP and D-[U-¹⁴C]glucose 6-phosphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The Polytron homogenizer was supplied by Northern Media Supply Ltd., North Cave, North Humberside, U.K.

Hanks buffer

Hanks buffer contained 121.6 mM-NaCl, 5.4 mM-KCl, 1.3 mM-CaCl₂, 0.8 mM-MgSO₄, 3.4 mM-Na₂HPO₄, 0.4 mM-KH₂PO₄, 4.2 mM-NaHCO₃, 4.2 mM-sodium pyruvate, 5.4 mM-sodium fumarate and 5 mM-sodium glutamate. This mixture was gassed with O₂/CO₂ (19:1); 0.2% (w/v) bovine serum albumin was added and the solution adjusted to pH 7.4 with 4 M-NaOH. The osmolarity was 310 mosm.

Preparation of cells

Adipocytes were prepared by collagenase digestion of rat epididymal fat-pads from 130–150 g rats by the method of Rodbell (1964), as described by Luzio (1979).

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).

Lymphocytes were isolated from two or three rat spleens removed from 250 g rats anaesthetized with diethyl ether. The spleens were washed in Hanks buffer at room temperature containing 0.1% heparin (sodium salt), chopped with scissors and forced through a steel-mesh wire tea strainer. The cells were collected in 20 ml of Hanks buffer containing heparin and centrifuged for 4 min at 100g on a bench centrifuge. The pellet was resuspended in 20 ml of 0.9% (w/v) NH₄Cl at pH 7.2, decanted to remove any lumps of tissue and centrifuged as above. The cells were then washed three times in Hanks buffer by a similar procedure and resuspended at a suitable concentration. Approx. 6×10^8 cells were isolated from two rats.

Preparation of antisera

Rabbit anti-(rat liver 5'-nucleotidase) serum was prepared by purifying 5'-nucleotidase from rat liver to a specific activity of approx. 60 units/mg of protein by the method of Widnell (1975), emulsifying this material with an equal volume of Freund's complete adjuvant and administering by multiple intradermal injection to New Zealand White rabbits. Each animal was injected monthly with approx. 200 μ g of protein. The rabbits were bled from an ear vein 10 days after the third and subsequent monthly injection; 1 ml of antiserum was capable of neutralizing 0.1 unit of 5'-nucleotidase from each cell type after incubation for 30 min at 37°C. It did not inhibit non-specific phosphatase (Newby, 1979). Fab fragments of this antiserum were prepared from an immunoglobulin G fraction of antiserum from which antibodies reacting with the erythrocyte surface had been removed. This was achieved by incubating 1 ml of fresh packed rat erythrocytes with 2 ml of serum at 4°C for between 4 and 16 h. Cells and serum were then separated on a bench centrifuge. After four absorptions the binding of the rabbit anti-(rat liver 5'-nucleotidase) serum to erythrocytes detected by using ¹²⁵I-labelled anti-(immunoglobulin G) (Hales, 1972) had been decreased 30–40-fold without affecting the titre towards purified 5'-nucleotidase. Fab fragments were prepared from this material by papain digestion followed by gel filtration as described by Porter (1959). By SDS/polyacrylamide-gel electrophoresis (Neville, 1971) at least 90% of this Fab preparation was of mol.wt. < 30000.

Rabbit anti-(rat erythrocyte) serum was prepared as described by Luzio *et al.* (1976).

Chicken anti-(rat liver enolase) serum was a generous gift from Dr. C. B. Taylor, Department of Biochemistry, University of Sheffield; 1 ml of this antiserum was capable of neutralizing 2.5 units of enolase activity (Rider & Taylor, 1974).

All antisera were heated to 56°C for 30 min before use to inactivate complement.

Homogenization of cells

Method 1: complete disruption of cells for the measurement of total 5'-nucleotidase activity. In experiments to determine the size of the intracellular pool of 5'-nucleotidase, it was observed that different methods of cell disruption which gave total release of the cytosolic marker enzyme lactate dehydrogenase did not always increase 5'-nucleotidase activity by a constant amount. In early experiments with hepatocytes, Polytron homogenization for 60s at setting 7 was sufficient to give a maximum increase in activity, but, as the instrument aged, addition of 0.5% detergent (either Triton X-100 or Lubrol 12A9) was also required. Detergent solubilization in 2% Lubrol without mechanical cell breakage always gave total release of enzyme activity, and this was later standardized as the method for disrupting hepatocytes and was also used to disrupt lymphocytes and adipocytes.

Method 2: damage to the hepatocyte plasma membrane without loss of intracellular membranes. Hepatocytes in Hanks buffer were placed in a loose-fitting silicone-treated Teflon/glass Dounce homogenizer (radial clearance 0.10mm) and given a small number of strokes (between 1 and 12). The method destroyed the permeability barrier of the hepatocyte plasma membrane, as measured by Trypan Blue exclusion and lactate dehydrogenase release, without extensive damage to intracellular structures.

Method 3: isolation of intact intracellular vesicles from hepatocytes. Isolation of intact intracellular vesicles was attempted by sucrose-density-gradient sedimentation. The Dounce homogenization was unsuitable for these experiments, since it caused little cell breakage when the hepatocytes were suspended in 0.25 M-sucrose containing 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. Cells were therefore homogenized by ten strokes in a close-fitting Potter homogenizer (radial clearance 0.05mm) rotating at 1500 rev./min. This procedure resulted in approx. 40% of the total cell 5'-nucleotidase being recovered in a post-mitochondrial (10000g, 10min) supernatant.

Assays

5'-Nucleotidase was assayed by the method of Avruch & Wallach (1971) as modified for whole cells by Newby *et al.* (1975). Since most cells contain many phosphatases, 20 mM-sodium β -glycerophosphate was included in all assays so that 5'-nucleotidase activities were measured as specific 5'-nucleotide phosphohydrolases. Assays each contained a total of approx. 5000 Bq (200000 c.p.m.) of [3 H]AMP and were linear with time up to 10% conversion of substrate.

Glucose 6-phosphatase (EC 3.1.3.9) was measured by the method of Kitcher *et al.* (1978). For this 400 μ l

of sample was incubated with 100 μ l of 50 mM-D-[U- 14 C]glucose 6-phosphate (in Hanks buffer) for 40 min at 37°C; approx. 5000 Bq (200000 c.p.m.) was added to each assay. The reaction was terminated by the addition of 300 μ l of 0.15 M-ZnSO₄ and the sample was then processed as for the 5'-nucleotidase assays.

Arylsulphatase (EC 3.1.6.1) was assayed by the method of Roy (1953). For this 450 μ l of the sample was incubated with 50 μ l of *p*-nitrocatechol sulphate (7.5 mM final concn.) in 0.1 M-sodium acetate buffer, pH 5.4 (final), for 30 min at 37°C. The reaction was terminated by adding 500 μ l of 4% (w/v) quinol in 2.5 M-NaOH containing 2.5% (w/v) Na₂SO₃. *p*-Nitrocatechol formation was measured from the *A*₅₁₅.

Lactate dehydrogenase (EC 1.1.1.27) was measured from the rate of NADH oxidation in the presence of 1.0 mM-sodium pyruvate (Keiding *et al.*, 1974).

Enolase (EC 4.2.1.11) was measured by the method of Shonk & Boxer (1964).

A unit of enzyme activity is the amount that catalyses conversion of 1 μ mol of substrate/min at 37°C. Unless otherwise stated, results are expressed as means $\pm$ S.E.M. for the numbers of experiments in parentheses.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Samples of hepatocytes for ATP measurement were deproteinized by adding 143 μ l of 40% (w/v) trichloroacetic acid to 1 ml of cells and centrifuging for 2 min at 10000g. Then 100 μ l of 1 M-HCl was added to the supernatant and the trichloroacetic acid extracted with 4 $\times$ 5 ml of water-saturated ether. Residual ether was blown off in N₂ gas and the HCl neutralized with 1 M-NH₃. Then 50 μ l of this sample was analysed by high-pressure liquid chromatography (Anderson & Murphy, 1976). ATP was identified by its retention time compared with standards and by its removal after incubation with hexokinase and glucose.

Results

The cell-surface 5'-nucleotidase activity of a variety of cell types isolated from rat and the increase in activity that may be measured after homogenization or detergent disruption are shown in Table 1. Homogenization (Method 1) doubled the 5'-nucleotidase activity of collagenase-isolated hepatocytes, whereas both lymphocytes and collagenase-isolated adipocytes exhibited 70–80% of their total 5'-nucleotidase activity at the cell surface. With each cell type studied, over 90% of homogenate activity was sedimented by centrifugation at 100000g for 60 min, suggesting that both the intracellular and cell-surface enzyme was membrane-bound. The cell-surface activities at 37°C of hepatocytes, adipocytes and lymphocytes were 15.0, 0.5 and 0.8 pmol of adenosine/min per cell respectively.

Table 1. Subcellular distribution of 5'-nucleotidase in various rat cell types

Cells from various rat tissues were isolated and assayed for 5'-nucleotidase in iso-osmotic medium as described in the text. The activities of cell-surface 5'-nucleotidase in hepatocytes, adipocytes and lymphocytes were 6.7 ± 0.3 (15), 0.012 ± 0.001 (4) and 0.4 ± 0.01 (8) units/g dry wt. respectively. The cells were then homogenized (Method 1) so as to disrupt intracellular structures completely. Data are expressed relative to total 5'-nucleotidase in each cell type as means $\pm$ S.E.M. (no. of experiments).

Cell type	5'-Nucleotidase activity (% of total)	
	Cell surface	Exposed on homogenization
Hepatocytes	48 ± 2 (26)	52 ± 2 (26)
Adipocytes	75 ± 4 (3)	25 ± 4 (3)
Lymphocytes	79 ± 2 (15)	21 ± 2 (15)

The nature and properties of the intracellular membrane-bound pool of 5'-nucleotidase were studied further in hepatocytes. Although total cell breakage (homogenization Method 1) caused complete release of the intracellular pool of the enzyme, gentler methods of homogenization did not. By using a loose-fitting Dounce homogenizer (Method 2) it was found that the plasma membrane of hepatocytes could be damaged so as to allow access of previously non-penetrating reagents to the cytosolic compartment of the cell without causing a change in the cell morphology observed by light microscopy. By using such damaged cells it was possible to correlate the access of AMP to intracellular 5'-nucleotidase with the access of substrate to glucose 6-phosphatase and the release of soluble marker enzymes, thus providing information about the intracellular location of the enzyme. During progressive cell damage glucose 6-phosphatase activity towards exogenous substrate closely paralleled the release of lactate dehydrogenase (Fig. 1), as expected from the proposed location of the enzyme on the luminal surface of the endoplasmic reticulum with access to the cytosol via a specific glucose 6-phosphate carrier (Arion *et al.*, 1975; Gold & Widnell, 1976; Ballas & Arion, 1977; Nilsson *et al.*, 1978). In contrast, the 5'-nucleotidase activity towards exogenous AMP showed a slower increase than did glucose 6-phosphatase, even though the damaged cells would be expected to have a similar permeability to the two substrates. By using the intercept of 5'-nucleotidase activity at zero lactate dehydrogenase release as a true measure of cell-surface 5'-nucleotidase activity (42% of total), it was possible to calculate the increase in availability of intracellular enzyme to exogenous AMP by subtraction from the total activity (Fig. 1, broken line). This increase correlated closely with arylsulphatase release, suggesting that all of the intra-

Fig. 1. Latency of hepatocyte intracellular 5'-nucleotidase

Freshly prepared hepatocytes were suspended at a concentration of 3.7 mg dry wt./ml in Hanks buffer at 37°C. Portions (15 ml) of the cell suspension were given between one and twelve strokes in a silicone-treated (Teflon/glass) Dounce homogenizer (Method 2). 5'-Nucleotidase and glucose 6-phosphatase were assayed in each preparation. No further cell breakage, as measured by lactate dehydrogenase release, occurred during these assays. At the same time as these assays were being performed, 3 ml of the broken cell preparation was centrifuged at 1000g for 30s and the supernatant assayed for lactate dehydrogenase and arylsulphatase. Each assay was performed in quadruplicate; random errors were smaller than the symbols shown. Release or exposure of each enzyme activity after the various Dounce treatments is plotted against the corresponding lactate dehydrogenase release. 5'-Nucleotidase activity (○), glucose 6-phosphatase activity (▲) and released arylsulphatase activity (■) are expressed as a percentage of the total activity (17.5, 43.1 and 32.9 nmol/min per mg dry wt. respectively). Intracellular 5'-nucleotidase (●) was calculated from the data for total 5'-nucleotidase by extrapolating to zero lactate dehydrogenase release to obtain a value for the cell-surface activity (42%) and then expressing the increase over this cell-surface activity after each Dounce-homogenizer treatment as a percentage of the original intracellular 5'-nucleotidase (58%).

cellular 5'-nucleotidase activity made available during progressive cell damage may be attributed to the damage of intracellular vesicles with a similar fragility to lysosomes. Both cell-surface and intracellular 5'-nucleotidase appear therefore to have their active site located on the extracytoplasmic side of the membrane in which they are situated.

Previously it has been proposed that one can inhibit and hence define cell-surface enzyme activity with non-penetrating macromolecular reagents (DePierre & Karnovsky, 1974; Newby *et al.*, 1975). In an attempt to confirm the existence of an intracellular pool of 5'-nucleotidase activity in hepatocytes we therefore incubated isolated cells with a rabbit anti-(rat liver 5'-nucleotidase) serum under conditions where it was capable of inhibiting over 95% of homogenate activity. After the cells had been incubated with antiserum for 30min at 37°C and subsequently washed free of unbound antibody, the cell-surface activity was found to be inhibited by 83% (Table 2). However, homogenization of these cells revealed that the internal component of 5'-nucleotidase was also decreased by 52%. The partial inhibition of the intracellular 5'-nucleotidase could not be explained by cell breakage (measured by lactate dehydrogenase release), since this increased by only 5% during incubation with the antiserum. Neither was the inhibition explained by diffusion of inhibiting antibody across the cell membrane, since in some of these experiments chicken anti-(rat liver enolase) serum was also added to the isolated cells, but only inhibited 5% of the cytoplasmic enolase compared with 74% of the enzyme if the cells were first homogenized.

Inhibition of the intracellular pool of 5'-nucleotidase also occurred when hepatocytes were incubated

for 30min at 37°C with monovalent Fab fragments prepared from the immunoglobulin G fraction of the rabbit anti-(rat liver 5'-nucleotidase) serum or with concanavalin A (Table 2). Non-immune rabbit serum and rabbit anti-(rat erythrocyte) serum, which had previously been shown to cross-react with liver cells (Hales, 1972), did not inhibit either pool of 5'-nucleotidase.

A time course of the inhibition of hepatocyte 5'-nucleotidase activity by rabbit anti-(rat liver 5'-nucleotidase) serum at 37°C is shown in Fig. 2. The inhibition of cell-surface activity reached a maximum within 5 min, but intracellular activity was inhibited more slowly, with approx. 50% inhibited after 30 min. 5'-Nucleotidase assays of inhibited homogenates were linear, showing that no redistribution of antibody occurred after homogenization.

Inhibition of the two pools of hepatocyte 5'-nucleotidase by rabbit anti-(rat liver 5'-nucleotidase) serum at 37°C was not affected by the presence of the protein-synthesis inhibitor cycloheximide at 100 µg/ml. The presence of drugs capable of disrupting cytoskeletal elements or of metabolic inhibitors decreasing cytoplasmic ATP concentrations similarly had no effect on antibody inhibition of the two pools of enzyme (Table 3).

The only effective way of preventing inhibition of the intracellular pool of 5'-nucleotidase was to cool

Table 2. Inhibition of cell-surface and intracellular 5'-nucleotidase

Hepatocytes (15ml, containing 0.6mg dry wt./ml) were incubated for 30min at 37°C with serum as shown at a dilution of 1:50, or concanavalin A at 58 µg/ml. The cells were then sedimented by centrifugation at 1000g for 30s, washed twice in 10ml of Hanks buffer and then resuspended in 15ml. A 10ml portion was homogenized (Method 1) and the 5'-nucleotidase activity assayed in both the whole-cell suspension and the homogenate. Intracellular 5'-nucleotidase was obtained by difference. All values are expressed as a percentage of the total cell 5'-nucleotidase activity in the absence of inhibitor, as means ± S.E.M. (no. of experiments).

Addition	5'-Nucleotidase activity (% of total)			
	Cell surface		Intracellular	
None	48 ± 2	(26)	52 ± 2	(26)
Anti-(5'-nucleotidase) serum	8 ± 1	(20)	25 ± 2	(20)
Concanavalin A	8 ± 1	(9)	24 ± 2	(9)
Anti-(5'-nucleotidase) Fab fragment	11 ± 1	(3)	36 ± 4	(3)
Anti-erythrocyte serum	48 ± 1*	(2)	54 ± 13*	(2)
Non-immune serum	45 ± 1	(3)	53 ± 11	(3)

* Range of values.

Fig. 2. Inhibition of hepatocyte 5'-nucleotidase
Hepatocytes (1ml, containing 5mg dry wt.) were incubated with rabbit anti-(rat liver 5'-nucleotidase) serum for the times shown. Hanks buffer (15ml) at 0°C was then added and the cells were immediately sedimented by centrifugation at 1000g for 30s. The cells were washed twice in 10ml of Hanks buffer and then resuspended in 15ml. A 10ml portion of this suspension was homogenized (Method 1) and 5'-nucleotidase assayed in both the cell suspension (○) and homogenate. Intracellular 5'-nucleotidase (●) was calculated by difference. Each value is expressed as a percentage of the uninhibited cell homogenate 5'-nucleotidase activity (14.2nmol/min per mg dry wt.).

the hepatocytes to 0°C. Incubation of cooled hepatocytes (15ml, containing 9mg dry wt.) with rabbit anti-(rat liver 5'-nucleotidase) serum (at a dilution of 1:50, v/v) for 2h at 0°C resulted in $91 \pm 6\%$ (4) inhibition of the cell-surface enzyme without significant inhibition of the intracellular enzyme. A similar result was achieved by incubating hepatocytes for 2h at 0°C with concanavalin A (Table 4). Further incubation of concanavalin A-inhibited cells for 16h at 0°C with α -methyl D-mannoside completely restored the cell-surface 5'-nucleotidase activity (Table 4).

The ability to inhibit selectively cell-surface 5'-nucleotidase by incubation of hepatocytes with antiserum at 0°C for 2h or at 37°C for short times (<5min; Fig. 2) allowed the investigation of the properties of the intracellular enzyme in a hepatocyte preparation where the cell surface contributed little 5'-nucleotidase activity. Fig. 3 shows a Lineweaver-Burk plot of the kinetics with respect to AMP of

5'-nucleotidase in a homogenate in which 96% of the enzyme activity was derived from the intracellular pool of enzyme. This plot was not significantly different from that of the cell-surface enzyme measured in intact uninhibited hepatocytes (Fig. 3), and allowed calculation of a K_m (39 μ M in the presence of 1 mM-adenosine) for both intracellular and cell-surface enzyme.

To identify further a population of intracellular vesicles containing latent 5'-nucleotidase, homogenate fractions from uninhibited and cell-surface-inhibited hepatocytes were centrifuged through sucrose density gradients. The cells were homogenized by Method 3 and centrifuged at 10000g for 10min. A portion of the supernatant was loaded on linear 15–60% (w/v) sucrose gradients, which were then centrifuged at 67000g for 24h. Fig. 4 shows the 5'-nucleotidase activity on these gradients assayed in the presence and absence of detergent. The gradients from both control and surface-inhibited

Table 3. *Effect of inhibitors on hepatocyte 5'-nucleotidase*

Hepatocytes (15ml, containing 0.6mg dry wt/ml) were preincubated with 20 μ g of cytochalasin B/ml and 0.1 mM-colchicine or 0.01 mM-vinblastine sulphate for 30min at 37°C, or with 5 μ g of oligomycin/ml and 5 mM-2-deoxy-D-glucose for 15min at 37°C as shown. Antiserum was then added to some incubations without washing the cells, which were incubated for a further 30min at 37°C. The cells were then washed in 2×10 ml of Hanks buffer and a 1 ml sample was taken into trichloroacetic acid and processed for ATP measurement. Some of the remaining cells were homogenized (Method 1) and 5'-nucleotidase activity was measured in cells and homogenate. Intracellular 5'-nucleotidase was obtained by difference. Each value represents the mean of four assays performed in one experiment. Activities are expressed as a percentage of the total homogenate activity in uninhibited cells. Abbreviation: n.m., not measured.

Preincubation	Incubation with anti-(5'-nucleotidase) serum	5'-Nucleotidase activity (% of total)		ATP (% of control)
		Cell surface	Intracellular	
—	—	41	59	100
—	+	6	33	91
Cytochalasin B+colchicine	+	5	34	n.m.
Cytochalasin B+vinblastine sulphate	+	7	33	n.m.
Oligomycin+2-deoxyglucose	—	51	49	2
Oligomycin+2-deoxyglucose	+	8	30	2

Table 4. *Inhibition of hepatocyte cell-surface 5'-nucleotidase with concanavalin A at 0°C*

Hepatocytes were isolated from rat liver by collagenase digestion and resuspended at 0.7mg dry wt/ml. Portions (15ml) of cells were cooled to 0°C and incubated with concanavalin A and subsequently with α -methyl D-mannoside as shown. All the cells were washed after both incubations in 2×10 ml of Hanks buffer at 0°C and then resuspended in 15 ml of cold buffer. A 10ml portion was removed and homogenized (Method 1) and the 5'-nucleotidase activity of cells and homogenates measured at 37°C. Each value is a mean percentage of the control homogenate activity (8.9nmol/min per mg dry wt.). Lactate dehydrogenase release was less than 2% during the experiment.

Concanavalin A (58 μ g/ml)	α -Methyl D-mannoside (50mM)	5'-Nucleotidase activity (% of total)		
		Cell surface	Intracellular	Ratio
—	—	46 $\pm$ 1 (5)	54 $\pm$ 3 (5)	1.2
2h at 0°C	—	9 $\pm$ 6* (2)	55 $\pm$ 2* (2)	6.1
2h at 0°C	16h at 0°C	40 $\pm$ 2 (4)	49 $\pm$ 2 (4)	1.2

* Range of values.

Fig. 3. Kinetics of cell-surface and intracellular 5'-nucleotidase

Freshly prepared hepatocytes were suspended at 4.1 mg dry wt./ml in Hanks buffer. A 1 ml portion of these cells was incubated with rabbit anti-(rat 5'-nucleotidase) serum (diluted 1:50) for 5 min at 37°C. The cells were then diluted with 15 ml of Hanks buffer at 37°C, washed once in a further 15 ml of buffer and finally resuspended in 20 ml. A 10 ml portion of this inhibited cell suspension was homogenized by Method 1. Control cells without antiserum were treated in a similar manner. The cell-surface 5'-nucleotidase was inhibited by 96% during this procedure, but the intracellular 5'-nucleotidase was inhibited by only 8%. Intact control cells or homogenates of inhibited cells were then incubated in iso-osmotic 5'-nucleotidase-assay medium containing different amounts of AMP and the rate of reaction was determined. Double-reciprocal plots were constructed from these data. No more than 4% of the substrate was consumed in any assay. Each point represents the mean of quadruplicate assays, the random error in these measurements being similar in size or smaller than the symbols shown. ○, Control cells (cell-surface 5'-nucleotidase); ●, inhibited homogenate (intracellular 5'-nucleotidase). The K_m for AMP in each case was 39 μ M (at 37°C in the presence of 1 mM-adenosine).

hepatocytes showed two peaks of 5'-nucleotidase activity. Peak I, which was not latent, probably represents 5'-nucleotidase solubilized during homogenization or left attached to small fragments of membrane. It could also represent a soluble 5'-

Fig. 4. Equilibrium sedimentation of hepatocyte post-mitochondrial supernatants

Hepatocytes (1 ml; 9 mg dry wt./ml) were incubated with rabbit anti-(rat liver 5'-nucleotidase) serum (diluted 1:50) for 5 min at 37°C. Then 15 ml of Hanks buffer was added and the cells were immediately centrifuged at 500g for 30s. The cells were resuspended in 15 ml of buffer and centrifuged twice. This resulted in 83% inhibition of cell-surface 5'-nucleotidase and 26% inhibition of the intracellular activity. Then 10 ml of 0.25 M-sucrose containing 10 mM-Hepes and 1 mM-MgCl₂ was added and the cells were homogenized by Method 3. This homogenate was centrifuged at 10000g for 10 min, and 4 ml of the supernatant (equivalent to 20% of the original 5'-nucleotidase activity) was loaded on a 20 ml 15–60% (w/v) sucrose density gradient containing 10 mM-Hepes and 1 mM-EDTA. After centrifugation for 24 h at 67000g the gradients were pumped out and fractions assayed for 5'-nucleotidase in the presence (●) and absence (○) of 0.1% Lubrol. Gradients were loaded with material derived from (a) uninhibited cells and (b) cells treated with antiserum.

nucleotidase activity, although the enzyme described by Fritzson (1968, 1969) and Van Den Berghe *et al.* (1977) would not be very active in the conditions of our 5'-nucleotidase assay. Arylsulphatase (presumably from broken lysosomes) and lactate dehydrogenase were also found in this region (results not shown). Peak II of 5'-nucleotidase was partially latent, suggesting the presence of sealed membrane vesicles. This region of the gradient contained glucose 6-phosphatase, but not arylsulphatase (results not shown). Whereas non-latent 5'-nucleotidase activity on the gradient was inhibited by 72% if hepatocytes were briefly (5 min, 37°C) incubated with inhibiting antiserum before homogenization, the latent activity was only inhibited by 47% (Fig. 4). These experiments suggest that hepatocytes

contain a population of vesicles with 5'-nucleotidase on the extracytoplasmic face, and that this enzyme is not inhibited by very short incubations (5 min, 37°C) of the intact cells with inhibiting antiserum.

Inhibition of hepatocyte cell-surface 5'-nucleotidase without inhibition of the intracellular pool allowed an investigation of the appearance of intracellular enzyme at the cell surface. Cells were incubated with rabbit anti-(rat liver 5'-nucleotidase) serum for 2 h at 0°C to give a high ratio of intracellular:cell-surface 5'-nucleotidase activity. After washing to remove the antiserum, these cells were incubated for a further 60 min at 37°C, and it was observed that 5'-nucleotidase redistributed from the intracellular pool to the cell surface, thus decreasing the ratio of intracellular to cell-surface activity towards that measured in control cells (Table 5). Since cycloheximide (100 µg/ml) was present throughout these experiments and there was no decrease in total cell enzyme activity during the 37°C incubation, the redistribution of enzyme cannot be due to synthesis or degradation, but instead suggests a continuous circulation of enzyme between the cell surface and the intracellular pool.

The increase in cell-surface 5'-nucleotidase activity shown in Table 5 implies that there was a net movement of enzyme to the cell surface from the intracellular pool equivalent to 4% of the cell-surface activity/h. This is not, however, a quantitative measurement of the rate of movement of enzyme to the cell surface, since it represents the difference between internalization of enzyme and appearance of enzyme at the cell surface. The ratio intracellular:cell-surface enzyme decreased from 4.8 to 2.8 in 1 h, approaching the control value of 1.0.

Constant movement of 5'-nucleotidase between the intracellular and cell-surface pools may explain the ability of antiserum and concanavalin A to inhibit both pools of enzyme when incubated with hepatocytes at 37°C (Table 2, Fig. 2). To test further the possibility that 5'-nucleotidase was continuously

appearing at the cell surface in these inhibition experiments, the kinetics of inhibition of intact hepatocyte activity by antiserum was compared with the inhibition of a homogenate from the same cells. Both substrate and antiserum were added to each incubation and samples were taken at 2 min intervals to measure the net conversion of [³H]AMP into adenosine over the following 30 min. Rates of reaction were computed from these data and are shown in Fig. 5. In order to slow down the inhibition of 5'-nucleotidase the antiserum was used at a dilution of 1:300. At this concentration in the presence of substrate the inhibition of homogenate 5'-nucleotidase was not complete. Nevertheless it was observed that the initial rate of inhibition in this homogenate was faster than that of whole cells. This observation is consistent with the appearance of intracellular 5'-nucleotidase at the cell surface in intact cells.

The uptake of concanavalin A into hepatocytes was investigated, since this acts as an inhibitor of 5'-nucleotidase in an analogous manner to the antibody (Table 2) and it is possible to displace bound concanavalin A with α-methyl D-mannoside (Table 4). When hepatocytes that had been treated at 37°C with concanavalin A were cooled to 0°C and incubated for 1 h with 100 mM-α-methyl D-mannoside, it was observed that only 62 ± 4% (3) of the original 5'-nucleotidase was recovered. If, however, the cells were solubilized in Lubrol before incubation with the α-methyl D-mannoside, 80 ± 8% (3) of the original activity was recovered. This suggests that during the preincubation at 37°C concanavalin A bound to 5'-nucleotidase was internalized to a site inside the cell such that it was no longer accessible to exogenous α-methyl D-mannoside at 0°C.

Discussion

In the present study we have shown that rat isolated hepatocytes, adipocytes and lymphocytes

Table 5. *Movement of intracellular 5'-nucleotidase to the cell surface*

Hepatocytes (1 ml, containing 10 mg dry wt./ml) were incubated with rabbit anti-(rat liver 5'-nucleotidase) serum for 2 h at 0°C or 5 min at 37°C. Both methods produced a differential inhibition of the cell-surface 5'-nucleotidase relative to the intracellular activity. The cells were then washed twice, resuspended in Hanks buffer and 5'-nucleotidase activity was measured in whole cells and homogenates over the next 60 min. Only small changes occurred in the homogenate activity during this time. These random errors have been removed by normalizing each homogenate activity to that measured at zero time (18 nmol/min per mg dry wt.). Intracellular 5'-nucleotidase was then calculated as the difference between cell-surface and homogenate activity. Each value is expressed as a percentage of the total uninhibited homogenate activity. The S.E.M. of 12 measurements in three experiments is shown.

Time (min)	Control cells			Inhibited cells		
	Surface	Intracellular	Ratio	Surface	Intracellular	Ratio
0	51 ± 1.9	49 ± 1.8	1.0	8 ± 1.2	38 ± 1.2	4.8
30	52 ± 2.3	48 ± 2.1	0.9	10 ± 0.7	36 ± 0.7	3.6
60	51 ± 2.5	49 ± 2.4	1.0	12 ± 0.3	34 ± 0.3	2.8

Fig. 5. Antibody inhibition of homogenate and cell-surface 5'-nucleotidase

Hepatocytes were prepared at a concentration of 0.6mg dry wt./ml and a portion was homogenized by Method 1. Portions (2ml) were then added in duplicate to 10ml of 5'-nucleotidase assay mixture in Hanks buffer. Samples (500 μ l) were removed from each incubation at 2min intervals and total adenosine production was measured by the standard 5'-nucleotidase assay. After 6min 40 μ l of rabbit anti-(rat liver 5'-nucleotidase) serum was added to each incubation and the progress of AMP conversion into adenosine followed for the next 30min. The rate of AMP hydrolysis was calculated from this time course, the mean deviation of experimental points from the fitted line being $1.1 \pm 0.2\%$. Cell-surface (●) and homogenate 5'-nucleotidase (■) are expressed as a percentage of the initial activity. In this experiment cell-surface and homogenate initial activities were 5.5 and 9.2nmol/min per mg dry wt. respectively.

contain an intracellular membrane-bound pool of 5'-nucleotidase activity in addition to activity expressed on the cell surface. In hepatocytes the intracellular pool of 5'-nucleotidase accounts for approx. 50% of the total cell activity and is present on the extracytoplasmic face of intracellular vesicles, which appear to have a susceptibility to mechanical homogenization similar to that of lysosomes. 5'-Nucleotidase activity latent to detergent can be isolated by sucrose-density-gradient centrifugation of hepatocyte homogenates and does not correlate with arylsulphatase activity, suggesting that at least some of the intracellular vesicles containing 5'-nucleotidase are not lysosomes. Both the intracellular

and cell-surface pools of membrane-bound 5'-nucleotidase have similar kinetic properties and probably represent different locations of the same enzyme. Our data obtained by using inhibiting antibodies and concanavalin A suggest that in hepatocytes there is movement of enzyme between the cell surface and intracellular vesicles independent of enzyme synthesis and degradation.

The definition and characterization of ectoenzymes relies mainly on the comparison of whole-cell and homogenate enzyme activities and the use of extracellular non-penetrating substrates and inhibitors (DePierre & Karnovsky, 1974; Trams & Lauter, 1974; Newby *et al.*, 1975). Our present experiments have exposed difficulties with both approaches.

The demonstration of an ectoenzyme by comparison of whole-cell and homogenate activities is clearly dependent on any intracellular enzyme being made available to substrate on homogenization. Using a Dounce homogenizer we were able to damage hepatocytes so that 70% of the lactate dehydrogenase was released into the medium while most of the intracellular 5'-nucleotidase remained latent to detergent. A similar result was obtained with adipocytes after homogenization by passing the cells through a steel mesh. No increase in 5'-nucleotidase activity was observed (Newby *et al.*, 1975), and there was little loss of latency of lysosomal marker enzymes (C. N. Hales & K. Docherty, personal communication). Simply using more severe mechanical homogenization may not expose all the activity either, since inside-out plasma-membrane vesicles may be generated at the same time as intracellular membranes are broken. The only rigorous approach is to solubilize the cells in detergent and verify independently that detergent does not activate the enzyme. It has been suggested (Emmelot *et al.*, 1964; Nakamura, 1976; Dipple *et al.*, 1978; Evans, 1978) that 5'-nucleotidase is activated by detergents, but this seems unlikely in the present experiments, since conditions were achieved where Lubrol and Triton caused no further increase in 5'-nucleotidase activity after severe mechanical homogenization. In addition, the least-dense peak of hepatocyte 5'-nucleotidase activity isolated on a sucrose gradient (probably representing non-vesicular enzyme) was not stimulated by detergents (Fig. 4).

The present experiments also show that care is required when defining ectoenzymes with non-penetrating inhibitors. Incubation of intact hepatocytes with either anti-(5'-nucleotidase) serum or concanavalin A at 37°C resulted in inhibition of both the cell-surface and intracellular pools of 5'-nucleotidase. Only by incubating the cells at 0°C were we able to inhibit solely the cell-surface activity. It is possible that after washing and warming the cells in order to measure 5'-nucleotidase activity some internalization of the inhibitor will occur;

however, the amount of inhibition should reflect the original amount of enzyme at the cell surface.

Inhibition of the intracellular pool of 5'-nucleotidase by incubation of hepatocytes with inhibiting antiserum or concanavalin A at 37°C suggested that a circulation of membrane-bound 5'-nucleotidase between the cell surface and the intracellular pool was possible. Internalization of enzyme-bound inhibitor at 37°C was proved in experiments which used the observation that concanavalin A may be removed from its binding sites by α -methyl D-mannoside. The appearance of intracellular 5'-nucleotidase at the cell surface was shown by incubating hepatocytes at 37°C after inhibition of the cell-surface enzyme at 0°C. The fact that appearance of intracellular 5'-nucleotidase at the cell surface was not caused by incubation of hepatocytes with anti-erythrocyte serum, which has been shown to cross-react with liver cells (Hales, 1972), showed that this circulation of 5'-nucleotidase was not simply a response to antibody binding to the cell surface and causing fusion of intracellular vesicles with the plasma membrane. Moreover, the observation that a monovalent Fab preparation from anti-(5'-nucleotidase) serum was almost as effective as native serum at inhibiting both pools of enzyme suggests that the circulation of 5'-nucleotidase in hepatocytes is not a process stimulated by a multivalent ligand, such as the capping of membrane antigens (Taylor *et al.*, 1971), but occurs continuously, more in the nature of membrane flow (Shaffer, 1965; Abercrombie *et al.*, 1970; Bretscher, 1976; Morré, 1977).

Although it was not possible to measure independently the rates of internalization and appearance at the cell surface of ecto-5'-nucleotidase, several properties of the process were determined. None of the observations made were affected by the presence of cycloheximide in the experiments, suggesting that the circulation of 5'-nucleotidase was independent of protein synthesis. In experiments where the cell-surface enzyme was inhibited and the cells were then washed and incubated at 37°C, no further loss of enzyme activity was seen, showing that enzyme circulation was independent of its degradation. The inhibition of the intracellular pool of enzyme by exogenous antiserum was only prevented by lowering the temperature to 0°C, and not by lowering the intracellular ATP concentration or by disruption of the cytoskeleton. It is of considerable interest that similar properties have been reported for the process of micropinocytosis in hepatocytes (Allison, 1977).

It seems probable that the phenomena observed in the present experiments are analogous to the internalization of hepatocyte-surface-bound ligands seen by others. In particular, Roth (1978) showed the internalization of surface-bound ferritin-labelled concanavalin A, and Bergeron *et al.* (1979) showed the uptake of ¹²⁵I-labelled insulin, both with a similar

time course to our observed inhibition of intracellular 5'-nucleotidase by exogenous antiserum. With respect to the present work it is particularly striking that Bergeron *et al.* (1979) found intracellular ¹²⁵I-labelled insulin inside vesicles which were similar to lysosomes in appearance, but did not contain lysosomal enzymes.

Circulation of plasma membrane through the cell has previously been suggested by Steinman *et al.* (1976) from morphological observations of pinocytosis in macrophages and L-cells, and by Tulkens *et al.* (1977), who studied the internalization of plasma-membrane-bound antibodies in fibroblasts and their subsequent re-appearance at the cell surface. The role of plasma-membrane movement through the cell is not clear, though it has been proposed to explain cell locomotion (Shaffer, 1965; Abercrombie *et al.*, 1970), is perhaps required for the expression of hormone activity (Varga *et al.*, 1976; Conn *et al.*, 1978), and may be involved in the repair of membrane components (Doyle *et al.*, 1978) and the degradation of extracellular proteins (Tanabe *et al.*, 1979). In hepatocytes it is possible that the circulation of 5'-nucleotidase reflects a movement of membrane between different domains of the cell surface which remain present on the surface of collagenase-isolated cells (Wisher & Evans, 1977), and that this in turn is responsible for the transport of material from the blood to bile *in vivo* (Jackson *et al.*, 1978; Orlans *et al.*, 1978).

The movement of 5'-nucleotidase between the cell surface and intracellular pools appears to occur in other cell types. Edelson & Cohn (1976) proposed endocytotic internalization of the enzyme as a route to degradation in macrophages. A circulation of the enzyme between the fibroblast cell surface and an intracellular pool that occurs independently of synthesis and degradation has been observed by Widnell (1979). Finally, we have shown a very rapid internalization of cell-surface 5'-nucleotidase in lymphocytes, which when incubated with exogenous inhibiting antiserum double their intracellular pool of enzyme within 30s (Stanley *et al.*, 1979). It is likely that our observation of cell-surface and intracellular pools of membrane-bound 5'-nucleotidase in three rat cell types is true of many cells and that movement of enzyme between these pools can occur independently of synthesis and degradation.

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