Characterization of different molecular forms of 5'-nucleotidase in normal serum and in serum from cholestatic patients and bile-duct-ligated rats

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1. Serum 5'-nucleotidase in rat and man is derived from the plasma membrane rather than the cytosol by the criteria of inhibition with $[\alpha\beta\text{-methylene}]$ ADP and antisera. 2. In individuals with cholestasis the serum enzyme is mainly present as a high- M_r form that in the presence of the zwitterionic detergent Sulphobetaine 14 has the electrophoretic characteristics of liver plasma-membrane ectoenzyme. A minor form of 5'-nucleotidase in cholestatic serum and all the enzyme in normal serum appears to be half the molecular size of the liver plasma-membrane ectoenzyme. 3. 5'-Nucleotidase from both normal and cholestatic rat serum was found to contain a polypeptide chain of apparent M_r 70000 by immunoblotting techniques. 4. It is suggested that the major form of 5'-nucleotidase in cholestatic serum is an ectoenzyme dimer derived from liver plasma membrane. All of the enzyme in normal serum and some of the enzyme in cholestatic serum is present as an active monomer derived from the ectoenzyme dimer.

A concomitant rise in the serum concentration of intrinsic membrane enzymes alkaline phosphatase and 5'-nucleotidase is usually regarded as indicative of cholestatic disease in man (Brocklehurst *et al.*, 1976; Ellis *et al.*, 1978). The serum enzymes are thought to derive ultimately from hepatocytes possibly reaching the serum from bile by regurgitation (Bergan *et al.*, 1975). In patients with cholestasis, some of the serum 5'-nucleotidase may be present as high- M_r aggregates (Brocklehurst *et al.*, 1976), in the form of membrane fragments also containing alkaline phosphatase and termed 'koinozymes' (De Broe *et al.*, 1975).

In rat, further studies have shown the importance of conjugated bile salts in causing the release of intrinsic membrane enzymes from hepatocytes without the occurrence of cell lysis (Billington *et al.*, 1980; Barnwell *et al.*, 1983). Although the mechanism of release of such enzymes into bile is now better understood, less is known of the

relationship between the bile and serum enzymes. In the case of 5'-nucleotidase it has been shown that the normal serum enzyme has immunological difference from the bile enzyme (Mullock *et al.*, 1977). The major isoenzyme of 5'-nucleotidase in rat hepatocytes is present as an ectoenzyme located predominantly at the bile-canalicular front of the cell surface (Evans, 1976), but also circulating through the cell via endocytic pathways (Stanley *et al.*, 1980; Luzio & Stanley, 1983). This enzyme exists predominantly as a dimer of identical subunits of M_r 70000 each with a hydrophobic domain (Baillyes *et al.*, 1984). A cytoplasmic 5'-nucleotidase, also present in hepatocytes, can be distinguished from the ectoenzyme by its substrate specificity, molecular size, lack of hydrophobic domain, recognition by antibodies and the effects of inhibitors (Itoh, 1981; Worku & Newby, 1983).

In the present study the nature and properties of 5'-nucleotidase in serum and bile of rat and man were examined in normal individuals and during cholestasis. Polyacrylamide-gel electrophoresis under non-denaturing conditions was used to define both the molecular size and the charge of the enzyme.

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Experimental

Materials

All reagents used were of the highest grade available. Detergents, radiochemicals and reagents for polyacrylamide-gel electrophoresis were as previously described (Bailyes *et al.*, 1982).

Assay of 5'-nucleotidase

Radioassay was as previously described (Bailyes *et al.*, 1982). To assess inhibition a sample containing 0.5 munit (nmol/min) of 5'-nucleotidase was incubated with inhibitor for 1 h at 37°C before addition of the radioassay cocktail.

Serum and bile samples

Normal human serum was collected from volunteer laboratory workers. Serum from patients with cholestasis was obtained from those samples in the routine National Health Service laboratory showing both high alkaline phosphatase activity (>500 munits/ml, range 500–2200 munits/ml; normal range 30–135 munits/ml) and high 5'-nucleotidase activity (>20 munits/ml, range 20–410 munits/ml; normal range 3–7 munits/ml). Samples of human bile collected via a biliary cannula contained approx. 200 munits of 5'-nucleotidase/ml, equivalent to enzyme activity in the serum of the same patients.

Rat serum was prepared from blood obtained by cardiac puncture of 200–250 g Wistar rats under ether anaesthesia. Rat bile was collected after cannulation of the common bile duct of anaesthetized rats. Serum was also collected 72 h after ligation and subsequent transection of the common bile duct (Mullock *et al.*, 1977; Kamada & Calne, 1983). In normal rat serum 5'-nucleotidase activity was 40–50 munits/ml, in bile 500–650 munits/ml and in serum from bile-ligated rats 450–550 munits/ml.

All bile and serum samples were stored at 4°C and used within 4 weeks of collection. No noticeable loss of 5'-nucleotidase activity occurred in this time.

Liver membrane 5'-nucleotidase

5'-Nucleotidase was partially purified from human liver removed from the body not more than 5 h *post mortem* and stored for up to 3 months at –70°C before use. Enzyme purification was essentially as described for rat liver by Bailyes *et al.* (1981), resulting in a yield relative to homogenate of 10% and a specific activity of 10 units/mg of protein (320-fold purification).

Partially purified rat liver 5'-nucleotidase (specific activity 24 units/mg of protein) and

immunoaffinity-purified enzyme (specific activity 280 units/mg of protein) were prepared as previously described (Bailyes *et al.*, 1981, 1982).

Plasma-membrane fractions from human liver and rat liver were prepared essentially as described for rat liver by Nakamura (1976). The specific activity of 5'-nucleotidase in the preparations was for human membranes 0.14 unit/mg of protein and for rat membranes 0.36 unit/mg of protein.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis under non-denaturing conditions was carried out with rat 5'-nucleotidase as previously described (Bailyes *et al.*, 1982). The system used was System 2860 of Chrambach *et al.* (1976), which operates at pH 9.6.

Human liver 5'-nucleotidase had a higher pI (5.8–6.0) than the rat enzyme (pI 5.4), and thus different stacking and resolving conditions were required in native polyacrylamide-gel electrophoresis. The system used was System F of Newby *et al.* (1982), with a final pH of 10.2 in the stacking gel and pH 10.9 in the resolving gel.

On non-denaturing gels, bile and cholestatic serum samples used were 20 µl, normal serum samples 100 µl. The effect of Sulphobetaine 14 was assessed by treating samples with 2% (w/v) Sulphobetaine 14 before electrophoresis, and running with 0.1% (w/v) Sulphobetaine 14 present in the gels. 5'-Nucleotidase activity was determined in 2 mm gel slices and recovery of the enzyme after electrophoresis was over 90% unless otherwise stated.

Antisera, immunoabsorbents and immunoblotting

Mouse monoclonal antibodies to rat liver 5'-nucleotidase coded 5N4-2 and 5NE5 (Siddle *et al.*, 1981; Bailyes *et al.*, 1984) and rabbit antiserum to immunoaffinity-purified rat liver 5'-nucleotidase (Luzio & Stanley, 1983) were as previously described. Rabbit antiserum to human liver 5'-nucleotidase was prepared by using the injection schedule previously described (Stanley *et al.*, 1980) with the partially purified human liver enzyme as antigen.

Immunoabsorbents were prepared by coupling immunoglobulin G fractions to diazocellulose as described previously (Siddle *et al.*, 1981).

Purified rat liver 5'-nucleotidase and 5'-nucleotidase adsorbed from rat serum samples on an immunoabsorbent prepared from monoclonal antibody 5NE5 were electrophoresed on to a sodium dodecyl sulphate/polyacrylamide gel, transferred to nitrocellulose paper and detected with rabbit antibodies as previously described (Bailyes *et al.*, 1984).

Results

The molecular size of 5'-nucleotidase in the serum of patients with cholestasis was compared with that of enzyme in normal serum by using the technique of polyacrylamide-gel electrophoresis under non-denaturing conditions. Analysis of size and charge of the enzyme was carried out by measuring R_F values at different gel concentrations and constructing a Ferguson plot of $\log R_F$ versus gel concentration (Ferguson, 1964; Rodbard & Chrambach, 1971). This allowed calculation of charge density (Y_0) from the intercept, and from the slope of the plot (K_r , the retardation coefficient), by comparison with known protein standards, an estimate of molecular size and apparent M_r (Bailyes *et al.*, 1982; Newby *et al.*, 1982).

When serum from patients with cholestasis was electrophoresed into 9% (w/v) polyacrylamide gels under non-denaturing conditions, most of the enzyme did not easily enter the resolving gel, but in addition to the enzyme found at the top of the gel a fast-moving peak of enzyme activity, termed F, was observed (Fig. 1). In the presence of Sulphobetaine 14, a zwitterionic detergent capable of solubilizing membrane 5'-nucleotidase (Bailyes *et al.*, 1982), all the enzyme in the sera of cholestatic patients entered 9% (w/v) polyacrylamide gels, forming two discrete peaks of activity, one fast-moving, with the same R_F as F, and one slow-moving, termed S (Fig. 1). Although the slow-moving S peak of 5'-nucleotidase activity was the major peak in Sulphobetaine 14-treated patients' sera, accounting for $74 \pm 3\%$ (28) (range 58–88%) of total activity, none of this form of the enzyme was observed in normal serum, where only the fast-moving peak was found after electrophoresis in the presence or in the absence of Sulphobetaine 14 (Fig. 1 and Table 1).

Ferguson plot analysis of human cholestatic serum 5'-nucleotidase (Fig. 2) showed that both S and F forms of the enzyme had the same charge density but differed in size, the S form having a K_r equivalent to a geometric mean radius of 3.4 nm and apparent M_r 140 000, and the F form a geometric mean radius of 2.5 nm and apparent M_r 70 000 (Table 1). Normal serum 5'-nucleotidase electrophoresed as a fast-moving (F) peak in the

Fig. 1. Polyacrylamide-gel electrophoresis of human serum 5'-nucleotidase. Serum samples (normal, 100 μ l, $\circ$ and $\triangle$; cholestatic, 20 μ l, $\bullet$ and $\blacktriangle$) were electrophoresed into 9% (w/v) polyacrylamide gels under non-denaturing conditions either in the presence ($\bullet$ and $\circ$) or in the absence ($\blacktriangle$ and $\triangle$) of Sulphobetaine 14. Slices (2 mm) of the resolving gel were assayed for enzyme activity by radioassay. The stacking gel (not shown) contained at most 5% of the total enzyme activity in the absence of Sulphobetaine 14. $\uparrow$ marks the position of the ion front.

Table 1. Properties of human serum, bile and liver plasma-membrane 5'-nucleotidase					
Data are presented as means $\pm$ S.E.M. for three determinations unless stated otherwise. Serum samples for centrifugation were diluted 5-fold with 0.25 M-sucrose/1 mM-MgCl ₂ /10 mM-Tris/HCl buffer, pH 7.4.					
5'-Nucleotidase activity	Normal serum 3–7 munits/ml (28)	Cholestatic serum 20–410 munits/ml (42)		Bile 180–220 munits/ml (3)	Liver plasma membrane 140 munits/mg of protein
Inhibition (%) by:					
[α B-Methylene]ADP (200 μ M)	92 $\pm$ 2	90 $\pm$ 2		98 $\pm$ 2	98 $\pm$ 2
Rabbit antiserum (1:10)	94 $\pm$ 2	98 $\pm$ 2		95 $\pm$ 3	94 $\pm$ 2
Centrifugation (180 000 g for 90 min), activity in pellet	Not detectable	35 $\pm$ 5% (10) (all in S form)		Not determined	—
Electrophoretic forms in presence of sulphobetaine 14	F 100%	F	S 74 $\pm$ 3% (28)	S	S
Ferguson-plot analysis:					
Y_0	3.0 $\pm$ 0.5	3.0 $\pm$ 0.2	3.0 $\pm$ 0.2	2.8 $\pm$ 0.3	2.9 $\pm$ 0.2
K_r	0.070 $\pm$ 0.003	0.068 $\pm$ 0.002	0.100 $\pm$ 0.004	0.089 $\pm$ 0.005	0.090 $\pm$ 0.003

Fig. 2. Ferguson plot of serum 5'-nucleotidase from patients with cholestasis and from bile-duct-ligated rats. Serum samples (20 μ l) were run in the appropriate electrophoresis system at different gel concentrations under non-denaturing conditions in the presence of Sulphobetaine 14. For each gel concentration duplicate gels were measured, and the plot of R_F (logarithmic scale) versus gel concentration (%) was constructed from the results for three serum samples by using least-squares linear regression. The plots represent the fast-moving (F) fraction (● and ▲) and the slow-moving (S) fraction (○ and △) of serum 5'-nucleotidase (● and ○, human; ▲ and △, rat).

presence or in the absence of Sulphobetaine 14, and could not be distinguished from the cholestatic-serum F form by Ferguson-plot analysis (Table 1), nor when run in the same gel. Sulphobetaine 14-treated human liver plasma-membrane 5'-nucleotidase showed only a single peak on polyacrylamide-gel electrophoresis equivalent to the slow-moving S peak (Table 1). Only part of the bile enzyme entered a 9% (w/v) polyacrylamide gel in the absence of Sulphobetaine 14 and did not form a discrete peak, but in the presence of this detergent only the slow-moving S peak of activity was observed (Fig. 3 and Table 1).

Since the electrophoretically fast-moving (F) form of 5'-nucleotidase found in normal serum and also in the serum of cholestatic patients was not sedimented by ultracentrifugation and entered gels

Fig. 3. Polyacrylamide-gel electrophoresis of human bile 5'-nucleotidase

Bile samples (20 μ l) were electrophoresed into 9% (w/v) polyacrylamide gels under non-denaturing conditions in the presence (●) or in the absence (▲) of Sulphobetaine 14. Gel slices (2mm) were assayed for enzyme activity by radioassay. ↑ marks the position of the ion front.

in the absence of detergent, it was either a cytosolic form of the enzyme or a membrane enzyme that had lost its hydrophobic, membrane-binding, domain. The presence of cytosolic enzyme was excluded by inhibition with reagents that do not inhibit the cytosolic enzyme (Table 1; Worku & Newby, 1983).

The existence of a membrane-binding domain in serum 5'-nucleotidase may be demonstrated by the technique of charge-shift electrophoresis, which is based on the fact that only intrinsic membrane proteins bind detergent micelles (Helenius & Simons, 1977; Bailyes *et al.*, 1984). Since the electrophoretically fast-moving (F) form of the serum enzyme migrates close to the ion front, detergent conditions were chosen to result in decreased mobility towards the anode if this form of the enzyme had a hydrophobic domain. The electrophoresis of 5'-nucleotidase from the serum of a patient with cholestasis was compared in Sulphobetaine 14 alone, i.e. an uncharged detergent, and in a mixture of Sulphobetaine 14 and the cationic detergent cetyltrimethylammonium bromide (Fig. 4). In the presence of cetyltrimethylammonium bromide both the S and F forms of 5'-nucleotidase showed decreased mobility towards the anode, indicating that both forms of the enzyme retained a hydrophobic, membrane-binding, domain. Further confirmation of the presence of a membrane-binding domain on the S form of 5'-nucleotidase in serum and bile was obtained by performing charge-shift experiments in the presence of 0.1% Sulphobetaine 14 and 0.01% *N*-dodecylsarcosinate, an anionic detergent that caused increased mobility of the S form of enzyme towards the anode (results not shown).

Fig. 4. Charge-shift electrophoresis of human serum 5'-nucleotidase

Serum samples (20 μ l) from a patient with cholestasis were electrophoresed into 8% (w/v) polyacrylamide gels with either 0.1% Sulphobetaine 14 in the stacking and resolving gels (○) or 0.1% Sulphobetaine 14 plus 0.005% cetyltrimethylammonium bromide in the anode buffer and gels (●). Cetyltrimethylammonium bromide inhibited the enzyme, decreasing recovery from 90% to 35%. Gel slices (2mm) were assayed for enzyme activity by radioassay. Total recovery of loaded 5'-nucleotidase 92% (○) and 35% (●) respectively. $\uparrow$ marks the position of the ion front.

Fig. 5. Polyacrylamide-gel electrophoresis of rat serum 5'-nucleotidase

Serum samples (normal, ○ and △; bile-duct-ligated, ● and ▲) were electrophoresed into 9% (w/v) polyacrylamide gels in the presence (● and ○) or in the absence (▲ and △) of Sulphobetaine 14. 5'-Nucleotidase activity was measured by radioassay of 2mm gel slices. $\uparrow$ marks the position of the ion front.

5'-Nucleotidase in the serum of normal rats, bile-duct-ligated rats and rat bile was compared by polyacrylamide-gel electrophoresis under non-denaturing conditions. A similar pattern of 5'-nucleotidase activity on polyacrylamide-gel electrophoresis was found to that observed with human enzyme. When serum from bile-duct-ligated rats was electrophoresed into 9% (w/v) polyacrylamide gels, much of the enzyme did not enter the resolving gel, and that which did formed a fast-moving (F) peak of enzyme activity (Fig. 5). In the presence of Sulphobetaine 14 all the enzyme entered the resolving gel, forming an additional slow-moving (S) peak of enzyme activity (Fig. 5). Normal rat serum contained only a fast-moving peak (F) of enzyme activity, which entered the resolving gel in the presence or in the absence of detergent (Fig. 5). Rat bile 5'-nucleotidase was analogous to human bile enzyme, entering 9% (w/v) polyacrylamide gels only in the presence of Sulphobetaine 14 and forming a discrete slow-moving peak, S (results not shown).

Ferguson-plot analysis of the two forms of rat serum enzyme showed that they had the same charge density, but differed in size. The S form of

the enzyme was not significantly different from immunoaffinity-purified rat liver membrane 5'-nucleotidase (Baillyes *et al.*, 1982) with an apparent M_r of 165 000, whereas the F form had an apparent M_r of 80 000. The F form in normal rat serum could not be distinguished from the F form in serum from bile-duct-ligated rats either from Ferguson-plot analysis or when electrophoresed in the same gels (results not shown).

Inhibition of 5'-nucleotidase activity in serum from normal or bile-duct-ligated rats or in rat bile was over 92% with either 200 μ M-[$\alpha\beta$ -methylene]-ADP or polyclonal rabbit antiserum. The monoclonal antibody 5N4-2, which partially inhibits rat liver membrane 5'-nucleotidase (Siddle *et al.*, 1981), inhibited the enzyme in normal serum by $33 \pm 5\%$ ($n = 3$).

The size of the polypeptide chains of rat serum 5'-nucleotidase was investigated by adsorbing the serum enzyme on a non-inhibitory monoclonal antibody (5NE5) immunoabsorbent, running this enzyme into a sodium dodecyl sulphate/polyacrylamide gel, transferring on to nitrocellulose and immunoblotting with a polyclonal rabbit antiserum. The latter can detect both the major 70 000- M_r polypeptide of immunoaffinity-purified liver membrane enzyme, and also a minor 38 000- M_r polypeptide, which has lost an extracellular domain including the active site (Baillyes *et al.*,

Fig. 6. Immunoblotting of rat serum 5'-nucleotidase

Rat serum 5'-nucleotidase (normal, 8 ml, 320 munits; bile-duct-ligated, 0.5 ml, 260 munits) was at least $\geq 78\%$ adsorbed on 0.5 mg of monoclonal antibody (5NE5) immunoadsorbent (60 h, 4°C). After sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the protein was electrophoretically transferred to nitrocellulose. This was treated with polyclonal rabbit anti-(5'-nucleotidase) immunoglobulin G (20 μ /ml), and labelled bands were detected with immunoperoxidase-labelled second antibody. Track (a), 1 μ g of immunoaffinity-purified rat liver 5'-nucleotidase; track (b), normal rat serum enzyme; track (c), enzyme from bile-duct-ligated rat. The markers on the left show the positions of M_r standards.

1984). The two serum samples only contained the 70000- M_r polypeptide (Fig. 6).

Discussion

By using the technique of polyacrylamide-gel electrophoresis under non-denaturing conditions,

in the presence of zwitterionic detergent, two forms of 5'-nucleotidase were found in the serum of patients with cholestasis and in that of bile-duct-ligated rats. The slow-moving (S) form of the enzyme had characteristics indistinguishable from the enzyme found in bile and liver plasma-membrane fractions and in rat appeared to be identical with the active dimer of M_r 140000 that has been immunoaffinity-purified in previous studies (Baillyes *et al.*, 1982, 1984). The S form of the enzyme appeared to be naturally present in bile and cholestatic serum as high- M_r aggregates or complexes, agreeing with previous reports (De Broe *et al.*, 1975; Brocklehurst *et al.*, 1976).

Cholestatic serum, but neither bile nor liver membranes, contained in addition to the S form of the enzyme an electrophoretically fast-moving form of the enzyme, F. In serum of both rat and man this form of the enzyme appeared to be half the molecular size of the S form but to have the same charge. Normal serum from rat or man contained only the F form of enzyme. Inhibition studies, charge-shift electrophoresis and immunoblotting suggest that the F form of the enzyme is most probably a monomer derived from the plasma-membrane dimer without the involvement of proteolytic cleavage. Some evidence for a monomeric form of the ectoenzyme was previously reported after *N*-dodecylsarcosinate treatment of mouse liver membranes (Evans & Gurd, 1973). A report (Mullock *et al.*, 1977) that 5'-nucleotidase in normal rat serum was immunologically distinct from that found in serum of bile-duct-ligated rats could not be confirmed in the present experiments. However, it is not inconceivable that some antibodies that react with 5'-nucleotidase in its membranous environment cannot react with the free monomer.

We cannot rule out the possibility that 5'-nucleotidase found in normal serum derives from a tissue other than liver, or from a domain of the hepatocyte surface different from that releasing bile 5'-nucleotidase. However, the observation that the amount of the F form in serum increases during cholestasis in addition to the appearance of the S form, plus the similarity of many properties of the F form to both the S form and liver membrane enzyme, suggest this as the most likely source of all the serum enzyme. The S form of serum enzyme appears to be identical with that in bile, and previous work suggests that it is most likely that this enters the blood-stream by bile regurgitation when the bile duct is obstructed (Bergan *et al.*, 1975; Mullock *et al.*, 1977). However, the possibility of complex mechanisms, such as reversed endocytic flow through the hepatocyte, cannot be ignored (Desmet, 1975). The F form of the enzyme found in the blood is not present in bile, and thus if

it derives from this source of enzyme an additional mechanism to regurgitation is required for its formation.

The present study reinforces the view that ligation of the common bile duct of the rat provides a good model for human cholestatic disease (Mullock *et al.*, 1977). In addition, data presented provide further evidence that the shedding of membrane enzymes from the cell surface and their subsequent metabolism will prove as specific and interesting a biochemical pathway as the traffic of membrane enzymes and their processing inside the cell.

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