Clearance of Human *N*-Acetyl- β -hexosaminidases from Rat Circulation

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(Received 26 August 1977)

Human *N*-acetyl- β -hexosaminidases A and P are probably sialylated, since they are susceptible to neuraminidase attack. When infused into the circulation of a rat they are removed more slowly than the non-sialylated forms of the enzyme from tissue and urine.

N-Acetyl- β -hexosaminidases exist in human tissues and body fluids as a family of interrelated forms. The most extensive study has been made of the tissue forms, *N*-acetyl- β -hexosaminidases A and B, which have been purified to homogeneity and found to differ in their subunit composition (Srivastava & Beutler, 1976; Geiger & Arnon, 1976). *N*-Acetyl- β -hexosaminidase A found in normal urine is thought to arise in the kidney and shares many of the properties of *N*-acetyl- β -hexosaminidase A from this tissue (Dance *et al.*, 1969). The major form of *N*-acetyl- β -hexosaminidase found in normal serum, hexosaminidase As, is very similar to the tissue form of *N*-acetyl- β -hexosaminidase A, but differs in its behaviour on ion-exchange resins (Ikonne & Ellis, 1973) and on electrophoresis (Swallow *et al.*, 1974). The properties of *N*-acetyl- β -hexosaminidase P, produced in serum during pregnancy, are intermediate between those of hexosaminidases A and B (Stirling, 1972). However, despite their different physical characteristics, all these forms have similar catalytic activities and they are kinetically indistinguishable when assayed with synthetic substrates.

It would appear that the A forms of hexosaminidase found in serum and tissues are structurally related, as in Tay–Sachs disease the mutation is thought to affect the α -subunit (Srivastava & Beutler, 1976) and both serum and tissue forms of hexosaminidase A are totally absent.

All forms of *N*-acetyl- β -hexosaminidase are presumed to be glycoproteins, since they are adsorbed on to concanavalin A–Sephadex and can be eluted with α -methyl mannoside (Brattain *et al.*, 1977). Direct evidence for covalently bound carbohydrate has now been obtained from chemical analysis of purified placental hexosaminidases A and B (Geiger & Arnon, 1976; Lee & Yoshida, 1976). Neither of the tissue forms of *N*-acetyl- β -hexosaminidase is affected as regards electrophoretic mobility by treatment with neuraminidase (Carmody & Rattazzi, 1974). In contrast, incubation of serum hexosaminidases with neuraminidase causes a decrease in their net negative charge that can be demonstrated by their altered

behaviour on ion-exchange chromatography (Ikonne & Ellis, 1973) or electrophoresis (Swallow *et al.*, 1974).

There has been no detailed study of the serum hexosaminidases, but by analogy with other sialylated serum glycoproteins they are assumed to have a long half-life in circulation. In the only study of the kinetics of removal of hexosaminidase from the circulation of a human patient (Brady *et al.*, 1975), human urinary hexosaminidase was very rapidly removed and appeared mainly in the liver.

Similar infusion techniques have been used by Stahl *et al.* (1976) to study the behaviour of various purified rat hydrolases infused back into rats, and by Marinkovic *et al.* (1976), who investigated the uptake of human α -mannosidase from rat circulation after intravenous injection of the enzyme.

We have used this technique to investigate the relative rates of clearance of different forms of *N*-acetyl- β -hexosaminidase in an attempt to assess possible physiological significance of the relatively subtle differences between the various forms.

Experimental

Materials

Pooled third-trimester pregnancy serum and human liver, obtained *post mortem*, were stored at -15°C until required.

DEAE-cellulose was purchased from Whatman Chemical Co., Maidstone, Kent, U.K. CNBr-activated Sepharose 4B was supplied by Pharmacia (G.B.) Ltd., London W.5, U.K.

4-Methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside (4-MeUmbGlcNAc) was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Neuraminidase type VI (from *Clostridium perfringens*) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sagatal (pentobarbital sodium) was purchased from May and Baker, Dagenham, Essex, U.K.

All other reagents were of analytical grade and purchased from Fisons, Loughborough, Leics., U.K.

Enzyme purification

10 mM-Sodium phosphate buffer, pH 6.8, was used throughout the purification, and all procedures were carried out at 4°C unless otherwise specified.

For purification of the enzymes from liver, a 50% (w/v) homogenate of the thawed tissue was prepared in buffer and centrifuged at 12000g for 30 min. The supernatant was adjusted to 60% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and stirred overnight. Precipitated protein was centrifuged as before, redissolved in buffer and dialysed against buffer until sulphate was no longer detected in the diffusate. Pooled third-trimester pregnancy serum was dialysed overnight against phosphate buffer. Freshly voided urine was vacuum-dialysed against buffer, and the precipitate that formed was removed by centrifugation at 3000 rev./min (r_{av} , 2 cm) for 5 min on an MSE Super Minor bench centrifuge.

Ion-exchange chromatography

Samples prepared as described above were applied to a column (2.5 cm $\times$ 30 cm) of DEAE-cellulose equilibrated in buffer. The column was washed with buffer and the adsorbed material eluted with a gradient of 0–0.4 M-NaCl in 800 ml of buffer.

Affinity chromatography

2-Acetamido-*N*-(6-aminohexanoyl)-2-deoxy- β -D-glucopyranosylamine was prepared as described by Koshy *et al.* (1975).

Concentrated salt-free hexosaminidase fractions from DEAE-cellulose were applied to the affinity column pre-equilibrated in buffer. The column was washed with buffer and then with buffer containing 40 mM-GlcNAc to elute hexosaminidase B, or with buffer containing 40 mM-GlcNAc and 30 mM-NaCl to elute hexosaminidase A.

Active fractions were vacuum-dialysed against phosphate-buffered saline [10 mM-sodium phosphate (pH 6.8)/0.9% NaCl].

Neuraminidase treatment

Neuraminidase was dissolved in 50 mM-sodium phosphate/200 mM-citric acid buffer, pH 5.0, to give a concentration of 10 units/ml. Neuraminidase (15 μ l) was added to 600 μ l of enzyme preparation and incubated overnight at room temperature (15°C).

Cellogel electrophoresis

Electrophoresis on Cellogel was used to identify isoenzymes from DEAE-cellulose by their characteristic mobilities and to monitor the effects of neuraminidase treatment.

Gels were rinsed in water and soaked in 0.04 M-sodium phosphate buffer, pH 6.8, for 20 min. The gels were blotted on filter paper and samples were applied 5 cm from the cathode end with the applicator supplied by the manufacturers. Electrophoresis was carried out in 0.04 M-sodium phosphate buffer, pH 6.8, at 200 V (10 mA) for 1 h. Active bands were detected by laying filter paper over the gel and flooding this with 0.4 mM-4-MeUmbGlcNAc in phosphate/citrate buffer, pH 4.5. Fluorescence was viewed under u.v. light at 365 nm.

Enzyme assays

During purification, 20 μ l of solution containing the enzyme was incubated with 100 μ l of 0.4 mM-4-MeUmbGlcNAc in phosphate/citrate buffer, pH 4.5, for 10 min at 37°C. The reaction was stopped by adding 2 ml of 0.2 M-glycine/NaOH buffer, pH 10.2.

Assay of the enzyme in plasma samples from rats was performed by incubating 100 μ l of plasma with 100 μ l of buffered substrate for 30 min at 37°C. The reaction was stopped by adding 2 ml of glycine/NaOH buffer.

Fluorescence was measured on a Locarte fluorimeter (activation at 340–380 nm; detection at 440 nm) standardized with quinine sulphate (4 μ g/ml in 50 mM- H_2SO_4).

Infusion of enzymes

Male Sprague-Dawley rats (180–210 g body wt.) were anaesthetized by injecting them intraperitoneally with 5.8 mg of Sagatal/100 g body wt.

In each experiment an enzyme sample (500 μ l) was introduced through a cannula inserted in the left jugular vein of the rat. Samples of blood (400 μ l) were withdrawn when required into a syringe containing 100 μ l of 3.8% (w/v) sodium citrate through a cannula inserted in the right carotid artery. The blood volume of the rat was maintained by periodically infusing iso-osmotic saline through the jugular cannula.

Plasma was prepared from the citrated blood samples by centrifuging at 3000 rev./min at room temperature for 5 min on a bench centrifuge, and was used immediately for enzyme assays.

Results

All *N*-acetyl- β -hexosaminidase samples showed just one band of activity on Cellogel electrophoresis. Enzyme samples were diluted with iso-osmotic saline to give an expected increase of at least double the endogenous plasma hexosaminidase activity when 500 μ l of enzyme preparation was infused. For the purposes of these calculations the blood volume of a 200 g rat was taken to be 15 ml.

Incubation of *N*-acetyl- β -hexosaminidase A with

rat plasma for up to 1 h resulted in no decrease in the activity of the enzyme. Intravenous infusion of 15 μ l of neuraminidase diluted to 500 μ l with phosphate-buffered saline had no effect on the endogenous *N*-acetyl- β -hexosaminidase activity, or on the rate of uptake of *N*-acetyl- β -hexosaminidase A when it was infused at the same time.

After infusion, human liver *N*-acetyl- β -hexosaminidase A was removed very rapidly from rat circulation; initial activities in plasma (at 2 min) were higher than the expected maximum values, but activity fell rapidly to about 20% of the expected maximum within 15 min. This residual activity disappeared more slowly, and 5% of the infused activity was still detectable after 35 min. The kinetics of clearance of *N*-acetyl- β -hexosaminidase B closely resembled those of *N*-acetyl- β -hexosaminidase A (Fig. 1a). Over the first 15 min the infused activity fell to about 20% of the predicted maximum value, but thereafter clearance was slower than that of hexosaminidase A, and 10% of the infused activity remained after 35 min.

Brady *et al.* (1975) reported that purified human urinary *N*-acetyl- β -hexosaminidase A was rapidly cleared from circulation after injection into an infant totally deficient in *N*-acetyl- β -hexosaminidase. We observed a very rapid removal of human urinary *N*-acetyl- β -hexosaminidase from rat circulation (Fig. 1a). Within 1 min only 65% of the infused activity was detectable in the plasma and this fell to 1–2% within a further 20 min. The kinetics of uptake resembled those of the tissue hexosaminidases A and B, but the initial rate of clearance was even more rapid than that observed for the tissue forms.

Human serum *N*-acetyl- β -hexosaminidase behaved quite differently from the tissue and urinary forms on infusion (Fig. 1b). Within 1 min of infusion and over a total period of 40 min the detectable activity in the plasma was between 40 and 50% of that expected. This initial loss of activity appears to be due to inhibition of *N*-acetyl- β -hexosaminidase As by a factor in the rat blood, since even after very short incubations *in vitro* in whole blood, only 30–40% of the added activity could be detected. Incubation of *N*-acetyl- β -hexosaminidase As with plasma derived from citrated blood resulted in 40–50% loss of the added activity. No such inhibition occurred in the presence of citrate alone, nor did it occur on incubation of the tissue isoenzymes with blood or plasma.

After neuraminidase treatment the electrophoretic mobility of *N*-acetyl- β -hexosaminidase As was decreased and it moved as a band of mobility intermediate between those of *N*-acetyl- β -hexosaminidases A and B. The kinetics of clearance of neuraminidase-treated hexosaminidase As were very similar to that of the tissue isoenzymes. High initial activities decreased rapidly to a residual 30% activity that was cleared more slowly. Hexosaminidase As

Fig. 1. Clearance of *N*-acetyl- β -hexosaminidases (a) Clearance from rat circulation of purified human liver *N*-acetyl- β -hexosaminidases A (O) and B (Δ) and the A form of the enzyme from urine (□). Enzymes were infused into the circulation of anaesthetized male Sprague-Dawley rats (200g), and plasma prepared from samples of blood withdrawn at intervals was assayed for *N*-acetyl- β -hexosaminidase as described in the Experimental section. (b) Clearance from rat circulation of purified human *N*-acetyl- β -hexosaminidases As (●) and P (●) prepared from third-trimester pregnancy serum and a sample of As treated with neuraminidase (■).

taken from the ion-exchange column and treated with neuraminidase was not cleared after infusion, and a constant activity at 40% of that expected was observed, as for the untreated enzyme. It was presumed that contaminating glycoproteins present were also desialylated and compete with the enzyme for uptake.

On infusion, serum *N*-acetyl- β -hexosaminidase P did not behave like either the tissue forms of hexosaminidase or like *N*-acetyl- β -hexosaminidase As (Fig 1b). The infused activity was not inhibited as was hexosaminidase As, but was slowly cleared from the circulation over the 40 min period studied, and at the end of that time 80% of the infused activity remained.

Neuraminidase treatment of hexosaminidase P decreased its electrophoretic mobility to that of *N*-acetyl- β -hexosaminidase B, indicating that like hexosaminidase As, this enzyme is sialylated.

Discussion

Although they have different subunit compositions, *N*-acetyl- β -hexosaminidases A and B are almost identical in their kinetics of uptake from rat circulation. *N*-Acetyl- β -hexosaminidase A from urine behaved similarly, and this gives additional support to the suggestion that it is very similar to hexosaminidase A from tissues (Dance *et al.*, 1969).

N-Acetyl- β -hexosaminidases As and P are cleared slowly from rat circulation, and in this they resemble other glycoproteins found in plasma. Neuraminidase treatment changes the electrophoretic mobility of *N*-acetyl- β -hexosaminidases As and P, although it does not affect the tissue forms or hexosaminidase A from urine, and on subsequent infusion the desialylated form of the As enzyme is removed from circulation with kinetics similar to those of the tissue isoenzymes. We suggest that the presence of sialic acids on the serum hexosaminidases is a significant factor in maintaining them in the circulation, and that removal of this blocking group renders them susceptible to uptake, in common with their non-sialylated tissue counterparts.

Tissue *N*-acetyl- β -hexosaminidases have been ascribed a role in the catabolism of glycolipids (Sandhoff, 1970), glycoproteins (T. Bearpark, B. Fournet, J. Montreuil, G. Spik, J. L. Stirling & G. Strecker, unpublished work), and glycosaminoglycans (Thompson *et al.*, 1973). The function of *N*-acetyl- β -hexosaminidase As is at present unknown, but it is possible that hexosaminidase As has no catalytic function in the blood, since it seems to be inhibited specifically by a factor present in serum. It may be present in transit from the tissue in which it is produced to another site in the body, where it is utilized. Its inhibition would prevent attack on susceptible serum components. The behaviour of hexosaminidase P on infusion is consistent with that of other sialylated serum glycoproteins, and its

gradual increase in serum during pregnancy is likely to be due to a rate of secretion that is faster than its rate of removal from circulation.

No attempt has been made in the present study to determine the site of uptake of the *N*-acetyl- β -hexosaminidases, but it is probable that a large proportion enters the liver, as has been found for other asialoglycoproteins (Morell *et al.*, 1971). However, there are exceptions to this. Stockert *et al.* (1976) found that orosomucoid with terminal mannose residues was taken up by the kidney and not the liver. In addition there seems to be marked specificity of uptake in different cell types, and much may depend on the exact nature of the terminal sugar of the glycoprotein. Cultured fibroblasts will recognize and take up enzymes purified from fibroblasts, but the uptake of enzymes from different sources is much less efficient (Hickman *et al.*, 1974). Despite close similarities between particular isoenzymes of *N*-acetyl- β -hexosaminidase extracted from different tissues, it is possible that there are subtle structural differences, involving carbohydrate, that determine the eventual distribution of the enzyme in the body, a role for the carbohydrate portion of glycoproteins suggested by Winterburn & Phelps (1972).

Similarly, carbohydrate may be important in determining the distribution of an enzyme between lysosomes and the extracellular space. Evidence for the deranged balance between the two is found in I-cell disease (mucopolipidosis type II), where there is excessive production of extracellular forms with a corresponding lack of lysosomal enzymes (Ikonne & Ellis, 1973). The report by Strecker *et al.* (1976) that a sialidase is missing from the leucocytes of patients with I-cell disease points to a role for carbohydrates in determining the distribution of these enzymes in the body.

This work was supported by a grant from the Medical Research Council.

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