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Purification of Two Hexosaminidases from Human Kidney

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Hexosaminidase forms A and B were isolated from human kidney in a homogeneous state as demonstrated by electrophoretic and enzymic criteria. The enzymes were stable for at least 18 months when stored at −20°C in 0.025M-phosphate buffer, pH 6.5. The molecular weights of forms A and B were estimated by gel filtration to be 111000 ± 1500 and 114000 ± 1600 respectively. The molecular weights of hexosaminidase A and B subunits were determined by using polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Hexosaminidase A dissociated into one subunit with mol.wt. 68000. Hexosaminidase B dissociated into three subunits with mol.wts. 100000, 68000 and 37000 respectively, and one protein band of mol.wt. 140000. After treatment of hexosaminidases A and B with iodoacetic acid, the molecular weights of the carboxymethylated polypeptide subunits were also estimated. Carboxymethylated hexosaminidase A dissociated into one major subunit of mol.wt. 18000 and two other protein bands of mol.wts. 65000 and 100000. Carboxymethylated hexosaminidase B dissociated into one major subunit of mol.wt. 19000 and an additional band of mol.wt. 37000. The K_m of the enzymes for the synthetic substrate *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was 0.8mM. Both enzymes were inhibited or activated by various metal ions. Double pH optima for the enzymes were found at pH 4.5 and 4.8.

Human hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30), a lysosomal hydrolase (Robinson & Stirling, 1968; Dance *et al.*, 1969, 1970; Li *et al.*, 1973), exists in two forms. These isoenzymes can be separated into an acidic form A and a basic form B which are similar in their molecular weights, K_m values, pH optima and inhibition behaviours, but which differ in electrophoretic mobility and thermostability. The A and B forms of the enzyme are indistinguishable immunologically (Srivastava & Beutler, 1972; Carroll & Robinson, 1973). Carroll & Robinson (1974) have isolated a low-molecular-weight protein from human liver, which cross-reacts with antisera to hexosaminidases A and B. Hexosaminidases catalyse the hydrolysis of *N*-acetylgalactosamine or *N*-acetylglucosamine from β-glycosidic linkages. It was assumed that hydrolysis of the *N*-acetylgalactosamine moiety of ganglioside GM_2 [Cer-Glc-Gal (AcNeu)-GalNAc] was catalysed by a lysosomal hexosaminidase (Kolodny *et al.*, 1969; Okada & O'Brien, 1969). Deficiency of hexosaminidase A leads to accumulation of ganglioside GM_2 in various tissues (O'Brien *et al.*, 1971; Sandhoff *et al.*, 1971; Murphy & Craig, 1972). In classic Tay–Sachs disease (variant B), hexosaminidase A activity is deficient and ganglioside GM_2 is stored (Okada & O'Brien, 1969; Sandhoff, 1969; Murphy & Craig, 1972). In variant AB of Tay–Sachs disease, the activities of both isoenzymes are slightly increased when assayed with artificial substrates, but degradation of ganglioside GM_2 is decreased (Young *et al.*, 1970; Sandhoff *et al.*, 1971). In Sandhoff's disease (variant 0), both isoenzymes A and B are absent and ganglioside GM_2 is stored in the tissues (Sandhoff, 1969; Sandhoff *et al.*, 1971).

In order to study the physicochemical and kinetic properties of hexosaminidases A and B, we have purified both enzymes from normal human kidney to homogeneous proteins. In the present paper we describe procedures for the purification of these enzymes, and also their activation, inhibition and subunit structure.

Experimental

Materials

Normal tissues were obtained from the Department of Forensic Pathology of the University of Texas Health Science Center. The tissues were washed with 0.9% NaCl at 4°C and were stored frozen at −80°C until used. Substrates used in the enzyme assays, standard proteins, reagents for polyacrylamide-gel electrophoresis, chromatographic material were obtained as described by Marinkovic & Marinkovic (1976). *p*-Nitrophenyl *N*-acetyl-β-D-galactosaminide and *o*-nitrophenyl *N*-acetyl-β-D-

glucosaminide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 4-Methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucosaminide was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Sephadex G-100 and sulphapropyl-Sephadex C-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Iodo[^{3}H]acetic acid (100–300mCi/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. Other reagents were analytical grade. Human serum albumin was free of hexosaminidase activity.

Tissue

The tissue was washed, homogenized and centrifuged as described by Marinkovic & Marinkovic (1976).

Enzyme assay

Fresh homogenate was assayed for *N*-acetyl-β-D-hexosaminidase, α-D-mannosidase, β-D-mannosidase, β-D-galactosidase, α-D-glucosidase, β-D-glucuronidase, α-L-fucosidase and β-D-fucosidase activities. The substrate solutions used in assaying these enzymes were prepared as described by Marinkovic & Marinkovic (1976). A portion (0.1ml) of 2.4mM-*p*-nitrophenyl β-2-deoxy-D-glucopyranoside was hydrolysed in McIlvaine's (1921) citrate/phosphate buffer, pH4.5, with 2μl or 5μl of a 1:10 dilution of the hexosaminidase enzyme extract. The blank contained the same amount of substrate solution, but water instead of enzyme extract. After incubation at 37°C for 10min, the reaction was stopped by the addition of 0.1ml of 2M-NH_3, and diluted with water to a total volume of 1ml. The A_{400} of the liberated *p*-nitrophenol was read in a Perkin–Elmer spectrophotometer. The absorption coefficient for the *p*-nitrophenolate ion at 400nm was $18.1 \times 10^3 M^{-1}$ and was corrected with the blank as described by Armstrong *et al.* (1966). One unit of enzyme was defined as the amount of enzyme that hydrolyses 1μmol of substrate/min at 37°C. Specific activities were expressed as units/mg of protein.

Fluorescent enzyme assay

A portion (0.1ml) of 5mM-4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucosaminide, which had been dissolved in sodium phosphate/citric acid buffer (McIlvaine, 1921), pH4.4, was hydrolysed with 5μl or 10μl of a 1:10 dilution of the hexosaminidase enzyme extract. After 10min at 37°C the reaction was stopped by the addition of 0.9ml of 0.1M-glycine previously adjusted to pH10.5 with 0.2M-NaOH. Free 4-methylumbelliferone was determined with an Aminco–Bowman spectrophotofluorimeter by using excitation at 360nm and emission at 450nm. Protein concentrations were determined by the procedure of Lowry *et al.* (1951), with crystalline plasma albumin as the standard.

Carboxymethylation

Preparation of completely reduced and carboxy[^{3}H]-methylated hexosaminidase A and B. For structural studies the enzymes were dialysed for 4 days in a cold-room (4°C) against water and subsequently freeze-dried. Both enzymes (0.5mg) were dissolved in 7M-guanidine hydrochloride/0.01M-Tris/HCl buffer, pH 8.5 (2ml). Dithiothreitol (0.3mg in 60μl of water) was added and the solution kept for 1h at room temperature (20°C) (O'Donnell *et al.*, 1970). Then iodo[^{3}H]-acetic acid (50μl of an aqueous solution containing 1mCi/ml) was added and mixed well. After 30min 7.4mg of unlabelled iodoacetic acid was added and the pH was readjusted to 8.5 with Tris. After 20min the mixture was dialysed against water in a cold-room (4°C) for 2 days and freeze-dried.

Concentrations of protein samples and chromatographic procedures were described previously by Marinkovic & Marinkovic (1976).

Gel electrophoresis

Polyacrylamide-gel disc electrophoresis (Canalco Co.) was performed as described by Davis (1964). Enzyme activity was detected either by incubating the intact gel with the appropriate 4-methylumbelliferyl glycoside substrate at pH4.4 at 20°C and subsequently viewing the fluorescent band of liberated product under a u.v. lamp, or by cutting the gel into 2mm slices and incubating the homogenized slices with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and assaying as described above. Within 10min a yellow colour appeared in the region of the hexosaminidase A and B.

Molecular-weight determination

Gel filtration. For the determination of the molecular weight of hexosaminidases A and B, a column (98cm × 2.5cm diam.) of Sephadex G-100 was prepared in 0.05M-sodium acetate/acetic acid buffer, pH7.2, containing 0.1M-NaCl. The sample (10–15mg) was applied to the column and eluted in 3ml fractions with the equilibrating buffer at a flow rate of 21ml/h. The column was calibrated with the following standards (mol.wt. in parentheses); Blue Dextran (2000000); human γ-globulin (157000); bovine serum albumin dimer and monomer (138000 and 69000 respectively); ovalbumin (45000); α-chymotrypsin (22500). A plot of the elution volumes of the standards against the logarithms of their molecular weights was

used to estimate the molecular weight of the hexosaminidases A and B.

Subunit molecular weights. Molecular weights of the subunits were determined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate by the method of Shapiro *et al.* (1967) with 5% gels. The standards used for the calibration curve and the conditions for electrophoresis were described previously (Marinkovic & Marinkovic, 1976). The hexosaminidases A and B were incubated in 0.05M-sodium phosphate buffer, pH7.1, containing 8M-urea and 1% each of sodium dodecyl sulphate and 2-mercaptoethanol. After incubation at 37°C for 3h, a 10μl sample containing 0.2mg of protein was applied to the top of each gel, followed by 10μl of 40% (w/v) sucrose.

Molecular weights of labelled carboxymethylated subunits were determined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate by the method of Segrest & Jackson (1972) with 5%, 7.5% and 10% gels. The following standards, prepared as described by Marinkovic & Marinkovic (1976), were used for the calibration curve (molecular weights in parentheses): subunits of β-galactosidase from *Escherichia coli* (130000); bovine serum albumin (69000); ovalbumin (45000); myoglobin (17200); bovine haemoglobin, α- and β-chains (16000); cytochrome *c* (12400). Carboxy[^{3}H]methylated hexosaminidases A and B were incubated in sodium phosphate buffer, pH7.1, containing urea, sodium dodecyl sulphate and 2-mercaptoethanol, as listed above, and either incubated at 37°C for 3h or heated at 100°C for 5min. A 10μl sample containing 0.1mg of protein was applied to the top of each gel, followed by 10μl of 40% (w/v) sucrose.

K_m values

These were calculated by the double-reciprocal-plot method of Lineweaver & Burk (1934).

Purification of human hexosaminidase

The purification procedures from step 1 to 3, which include extraction, treatment with citric acid, concentration and $(NH_4)_2SO_4$ precipitation were described previously (Marinkovic & Marinkovic, 1976).

Step 4: *DEAE-cellulose chromatography.* The material from step 3 (300ml) was added to a column (37cm×5cm diam) of DEAE-cellulose type 40. The column was equilibrated and eluted with 0.01M-potassium phosphate buffer, pH6.0, at a flow rate of 80ml/h. Hexosaminidase B was not adsorbed and was eluted in fractions 31–150 (volume 9.5ml each). A linear NaCl gradient from 0 to 0.3M in the same buffer was then used to develop the column. Fractions with hexosaminidase A activity were eluted between 0.1M- and 0.25M-NaCl and were pooled. Recoveries from DEAE-cellulose of hexosaminidase A and hexosaminidase B were 98%. Fractions containing 78% of the hexosaminidase A activity were concentrated to 190ml and dialysed for 2 days against 0.01M-potassium phosphate buffer, pH6.0. Fractions containing hexosaminidase B were treated identically. From this step onward, the isoenzymes were purified separately.

Further purification of hexosaminidase A

Step 5: *DEAE-cellulose chromatography.* The concentrate of hexosaminidase A activity from step 4 was applied to a column (82cm×2.5cm diam.) of DEAE-cellulose type 40 by the procedure of Robinson & Stirling (1968) as described above.

Step 6: *CM-cellulose chromatography.* The fractions from step 5 containing enzyme-A activity were combined, and the volume was decreased to 200ml by ultrafiltration. The solution was dialysed against 0.01M-potassium phosphate buffer, pH6.0, and then applied to a column (85cm×2.5cm diam.) of CM-cellulose equilibrated with the same buffer. The hexosaminidase A was adsorbed and was eluted in fractions 100–180 (9ml each). A linear KCl gradient from 0 to 0.25M in the same buffer was then used to develop the column. Fractions with hexosaminidase A activity were eluted between 0.05M- and 0.2M-KCl and were pooled. These fractions were concentrated to 150ml by ultrafiltration, and the concentrate was dialysed against 0.01M-sodium acetate / acetic acid buffer, pH4.8.

Step 7: *Sulphopropyl-Sephadex C-25 chromatography.* The dialysed solution was centrifuged at 5000*g* for 20min in a Sorvall refrigerated centrifuge. The supernatant (150ml) with hexosaminidase A activity was applied to a column (33cm×2.5cm diam.) of sulphopropyl-Sephadex C-25 equilibrated with 0.01M-sodium acetate/acetic acid buffer, pH4.8. The hexosaminidase A was adsorbed and then eluted with a KCl gradient (0–0.3M) at 0.05M-KCl in the same buffer. Fractions 60–85 (each 8.5ml) were combined, decreased in volume to 25ml, and then dialysed against 0.01M-Tris/HCl buffer, pH7.70.

Step 8: *DEAE-cellulose chromatography.* The dialysed solution was applied to a column (20cm×2.2cm diam.) of DEAE-cellulose equilibrated with 0.01M-Tris/HCl buffer, pH7.70. The hexosaminidase A was adsorbed and was eluted with a KCl gradient (0–0.3M) in fractions 120–140 (4.6ml each), at 0.01M-KCl in the same buffer. These fractions were combined and concentrated to 10ml by ultrafiltration, and dialysed against 0.01M-sodium acetate / acetic acid buffer, pH4.8.

Step 9: *Sulphopropyl-Sephadex C-25 chromatography.* The hexosaminidase A fraction from step 8 was added to a column (10cm×2cm diam.) of

sulphopropyl-Sephadex C-25. The column was developed with a KCl gradient (0–0.2M) in 0.01M-sodium acetate/acetic acid buffer, pH4.8, and was eluted in fractions 70–90 (5ml each), at 0.05M-KCl. The active fractions were combined and concentrated to 1ml.

Step 10: *Gel filtration on Sephadex G-200.* The hexosaminidase A fraction from step 9 was subjected to gel filtration on a column of Sephadex G-200, equilibrated with 0.025M-potassium phosphate buffer, pH6.0. The hexosaminidase A was eluted with the same buffer in 3ml fractions at about tube 59. The active fractions were combined and dialysed against 1mM-potassium phosphate buffer, pH6.5.

Further purification of hexosaminidase B

Step 5: *CM-cellulose chromatography.* The dialysed solution with hexosaminidase B activity was applied to a column (80cm×2.5cm diam.) of CM-cellulose equilibrated with 0.01M-potassium phosphate buffer, pH6.5. The hexosaminidase B was adsorbed and the column was developed with a potassium phosphate gradient, 1 litre each of 0.01M, pH6.5, and 0.05M, pH8.5. The enzyme was eluted at pH7.10 and 0.02M-phosphate in fractions (10ml) around tube 90. The active fractions were combined and the volume was concentrated to 100ml by ultrafiltration. The concentrate was then dialysed against 0.01M-potassium phosphate buffer, pH6.0.

Step 6: *CM-cellulose chromatography.* The dialysed solution from step 5 with hexosaminidase B activity was applied to a column (86cm×2.5cm diam.) of CM-cellulose equilibrated with 0.01M-potassium phosphate buffer, pH6.0. The hexosaminidase B was adsorbed and was eluted with a KCl gradient (0–0.3M) in fractions 90–120 (10ml each), at 0.037M-KCl in the same buffer. These fractions were combined and concentrated to 50ml by ultrafiltration.

Step 7: *Hydroxyapatite column chromatography.* The hexosaminidase B fraction from step 6 was dialysed against three 1-litre changes of 0.01M-potassium phosphate buffer, pH6.5, and then applied to a column (10cm×2.2cm diam.) of hydroxyapatite equilibrated with the same buffer. The column was developed with a potassium phosphate gradient (0.01–0.5M) at pH6.5. The hexosaminidase B was eluted in 4ml fractions around tube 120, at 0.225M-potassium phosphate buffer. The fractions were combined for a total volume of 260ml.

Step 8: *Sulphopropyl-Sephadex C-25 chromatography.* The hexosaminidase fractions from step 7 were concentrated to 20ml and added to a column (31cm×2.5cm diam.) of sulphopropyl-Sephadex C-25. The hexosaminidase B was eluted in fractions (5ml) with a KCl gradient (0–0.3M) around tube 115 at 0.125M-KCl in 0.01M-sodium acetate/acetic acid buffer, pH4.8. Fractions 91–130 were combined and concentrated to 35ml by ultrafiltration and the concentrate was dialysed against 0.01M-Tris/HCl buffer, pH7.7.

Step 9: *DEAE-cellulose type-70 chromatography.* The dialysed solution with hexosaminidase activity was applied to a column (32cm×1.2cm diam.) of DEAE-cellulose 70 equilibrated with 0.01M-Tris/HCl, buffer, pH7.7. The hexosaminidase B was adsorbed and was eluted with a KCl gradient (0–0.3M) in fractions 120–160 (2.1ml each) at 0.1M-KCl in the same buffer. Fractions 128–140 were combined and concentrated to 1ml.

Results

Purifications of hexosaminidase forms A and B are summarized in Table 1.

Disc electrophoresis

Both purified hexosaminidases were subjected to electrophoresis at pH8.9 as shown in Plate 1(*a*). A single band of protein stain was detected. The enzymic stain of hexosaminidases A and B purified from human kidney has the same mobility as the enzymes from crude kidney extract in polyacrylamide-gel disc electrophoresis (Plate 1*b*).

Molecular weights of the enzymes and enzyme subunits

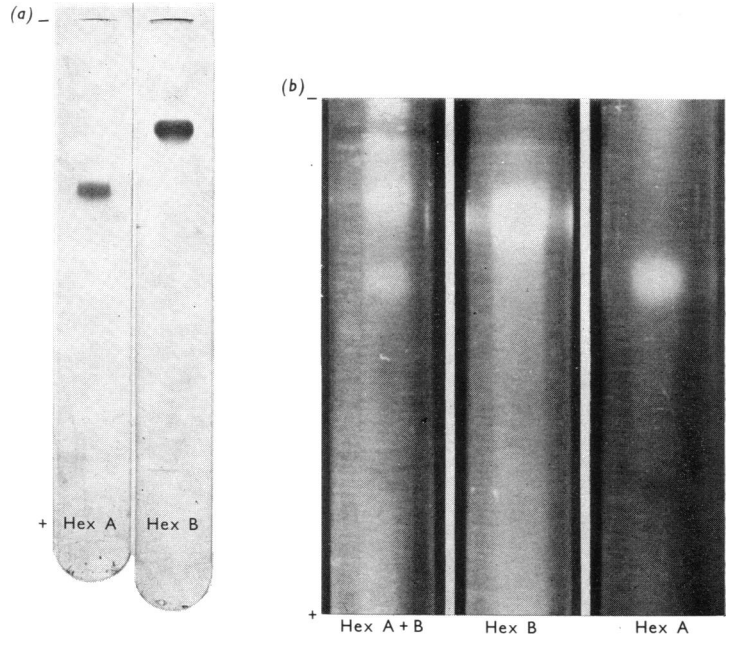
Gel filtration. The molecular weights of hexosaminidases A and B were determined by comparison with marker proteins run under identical conditions. Duplicate measurements of V_e/V_o agreed within 1%, which would introduce an error of about 3%. The gel-filtration data for marker proteins fitted to the following equation, obtained by the method of least squares:

$$\log M = 6.5087 - 1.2640 V_e/V_o \qquad (1)$$

The value of the molecular weights of hexosaminidases A and B obtained by using eqn. (1) were 111000±1500 and 114000±1600 respectively.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Additional tests of homogeneity and molecular weight were performed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins. Quadruplicate determinations were carried out on 5% gels. Purified hexosaminidases and marker proteins were treated with sodium dodecyl sulphate, 2-mercaptoethanol and 8M-urea and run on the gels. Hexosaminidase A dissociated into one band of mol.wt. about 68000. Trace amounts of a protein band of mol.wt. 140000 were also detected. Hexosaminidase B was



EXPLANATION OF PLATE 1

Polyacrylamide-gel disc electrophoresis of human kidney hexosaminidases A and B (Hex A and Hex B), (a) at pH 8.5, protein stain and (b) at pH 10.5, activity stain

(*a*) The gel was stacked at pH 8.5 with sample containing 10 μg of both hexosaminidase A and B and run at 3 mA/gel (Davis, 1964). The final hexosaminidases A and B after steps 10 and 9 respectively (purification) were applied. The gels were stained for protein with Coomassie Brilliant Blue R-250. (*b*) Enzyme activity was detected by incubation with 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as substrate followed by 0.05 M-glycine/NaOH buffer, pH 10.5. A fluorescent band developed at the place of enzyme activity.

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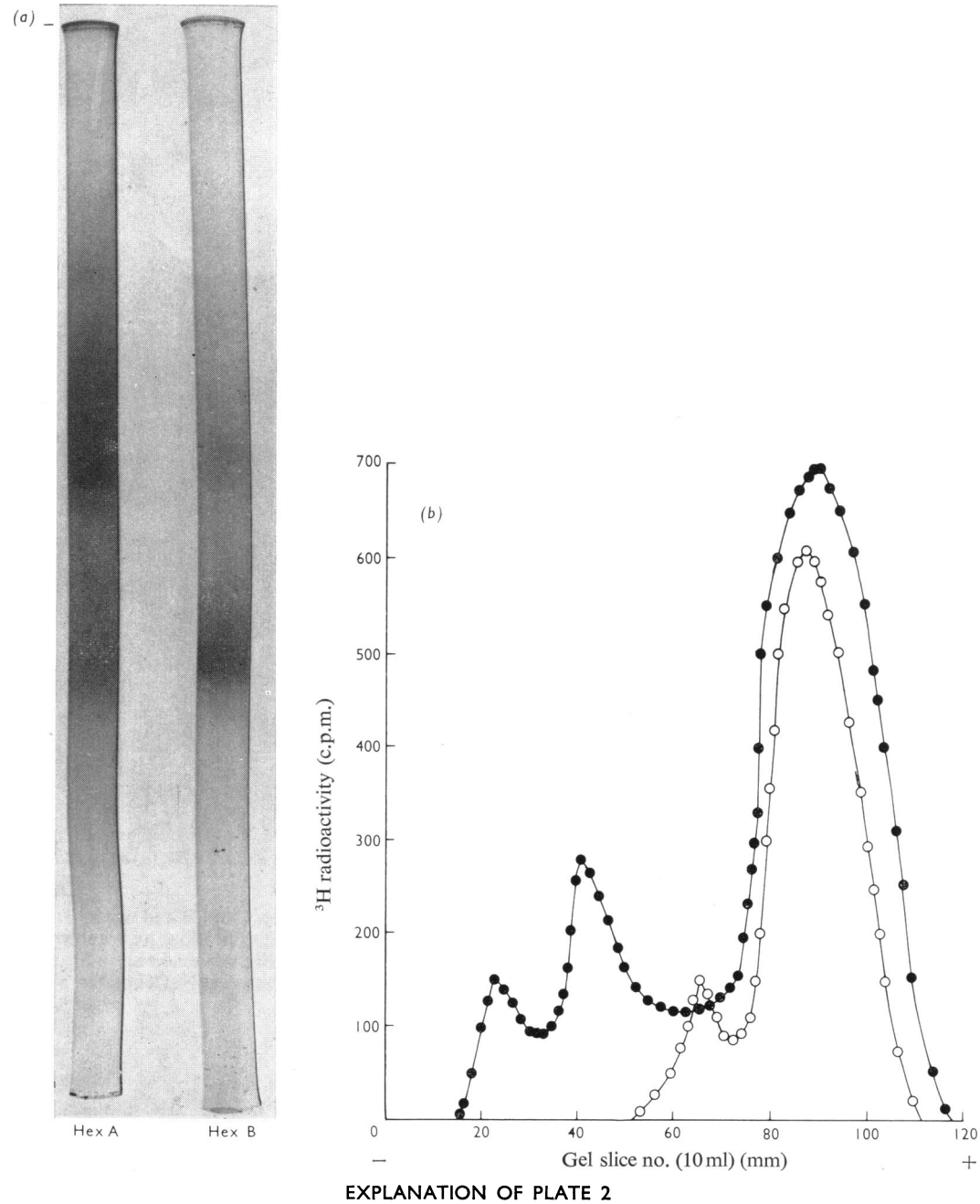

EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of ^{3}H-labelled subunits obtained by incubation of iodo[^{3}H]acetic acid with hexosaminidase A and B (Hex A and Hex B), for subunit molecular-weight determination

(*a*) Hexosaminidase A and B were treated with 7M-guanidine hydrochloride in Tris/HCl buffer, pH 8.5, and carboxymethylated as described in the Experimental section. Representative gels of carboxymethylated hexosaminidase A and B in 7.5% polyacrylamide gels are shown. The enzymes were heated at 100°C for 5min in 8M-urea and 1% each of sodium dodecyl sulphate and 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue R-250 in water, and destained by diffusion in 7% (w/v) acetic acid. (*b*) The preparation of labelled enzymes is described in the Experimental section. The analysis of the gels for radioactivity was carried out on 2mm slices. The slices were incubated at 55°C overnight in 0.5ml of 30% (v/v) H_2O_2, 10ml of scintillation fluid was added, and the samples were counted for radioactivity. ●, Hexosaminidase A; ○, hexosaminidase B.

Table 1. *Purification of human kidney N-acetyl-β-hexosaminidases*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield
Crude extract	84700	7143	0.084	100
Supernatant (pH4.5)	44150	6858	0.155	96
Amicon PM-30	28600	6850	0.239	95
$(NH_4)_2SO_4$ (65% satn.)	19454	6680	0.343	93
4. DEAE-cellulose 40 (pH6.0)				
Hexosaminidase A	11050	4755	0.430	66.5
Hexosaminidase B	6252	1284	0.205	17.9

Purification of hexosaminidase A

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield
5. DEAE-cellulose 40 (pH6.00)	4500	2298	0.510	32.0
6. CM-cellulose 32 (pH6.00)	793	1371	1.728	19.0
7. Sulphopropyl-Sephadex C-25 (pH4.8)	92	1026	11.15	17.36
8. DEAE-cellulose 70 (pH7.7)	70	854	12.20	11.95
9. Sulphopropyl-Sephadex C-25 (pH4.8)	11	344	31.27	4.81
10. Sephadex G-200 (pH6.0)	1.90	162	85.26	2.26

Purification of hexosaminidase B

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield
5. CM-cellulose 32 (pH6.50)	2270	990	0.436	13.85
6. CM-cellulose 32 (pH6.0)	1025	850	0.829	11.90
7. Hydroxyapatite gel (pH6.5)	187	520	2.78	7.27
8. Sulphopropyl-Sephadex C-25 (pH4.8)	24	400	16.66	5.59
9. DEAE-cellulose 20 (pH7.7)	1.5	147	93	2.05

resolved into at least four bands, with apparent mol.wts. about 37000, 68000, 100000 and 140000 respectively. These results are presented in Fig. 1. The same preparations of both enzymes were labelled with iodo[^{3}H]acetic acid. The carboxy[^{3}H]methylated enzymes were treated as described above, and electrophoresis was carried out on 5%, 7.5% and 10% gels (see the Experimental section for details). Three bands with mol.wts. 100000, 65000 and 18000 were obtained for hexosaminidase A. Hexosaminidase B dissociated into subunits with mol.wts. 37000 and 19000, as shown in Plates 2(*a*) and 2(*b*).

Enzymic properties of human kidney hexosaminidases

(1) *pH optimum.* The effects of pH on enzyme activity were studied by adjusting *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide to various pH values by using a wide-range buffer (0.1M-citric acid/0.2M-sodium phosphate). The hexosaminidases were then assayed for their activity as described in the Experimental section. Two pH optima were found for both enzymes, near pH4.5 and pH4.8 (Fig. 2). K_m of the purified enzymes for *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide was determined to be 0.8×10^{-3}M. The purified enzymes did not lose activity when stored at −20°C for at least 18 months at pH6.5.

(2) *Effects of inhibitors and activators on human kidney hexosaminidases A and B.* The effects of various metals on enzyme activity were examined by incubation of the purified enzymes with the ions for 30min at 25°C before the introduction of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide. Preincubation of both hexosaminidase A and B in the presence of 1mM-Hg^{2+}, -Ag^+ or -*p*-chloromercuribenzoate completely inhibited both enzymes, which suggests that thiol groups are part of the enzymes. Inhibition was also obtained with urea and sodium dodecyl sulphate. Table 2 lists the various compounds tested and their effects on the activity of both hexosaminidases of human kidney. No inhibition of hexosaminidase A and B was observed in the presence of 1mM-iodoacetate, -Zn^{2+}, -Na^+, -Mg^{2+} and -2-mercaptoethanol. Only human serum albumin substantially increased activity of hexosaminidase A and B.

The ratio of *N*-acetyl-β-D-glucosaminidase to *N*-acetyl-β-D-galactosaminidase activity obtained for the kidney enzymes was about 8:1, and is the same as

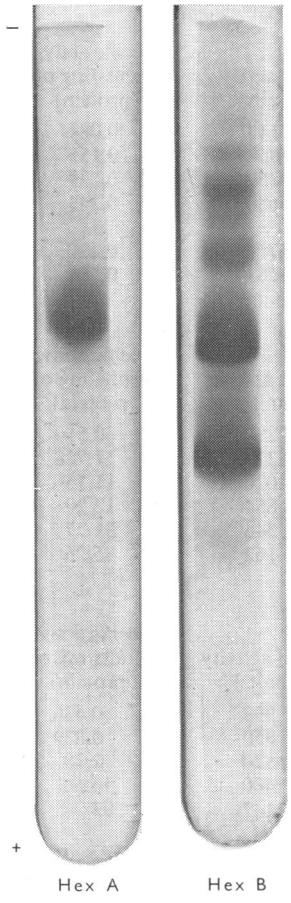

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of hexosaminidases A and B (Hex A and Hex B) at pH7.1*

The enzymes (0.2mg) were dissolved in 0.05M-sodium phosphate buffer, pH7.1, containing 8M-urea and 1% each of sodium dodecyl sulphate and 2-mercaptoethanol, and incubated at 37°C for 3h. The enzymes were subjected to electrophoresis at 6mA/gel for 3h at room temperature.

found for liver enzymes by Carroll & Robinson (1973). However, the ratios of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminidase to *o*-nitrophenyl *N*-acetyl-β-D-glucosaminidase activity for kidney hexosaminidases A and B were 3.3:1 and 5.5:1 respectively.

Discussion

In this paper we describe a procedure for the purification of β-hexosaminidase A and B from normal human kidney. After about 1000-fold purification, both purified enzymes appeared homogeneous by

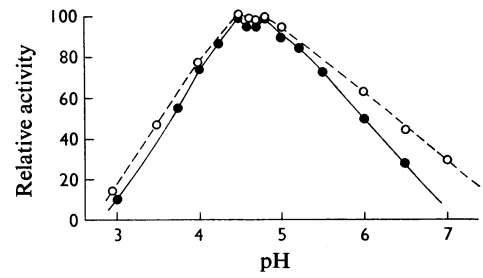

Fig. 2. *Effect of pH on activity of human kidney hexosaminidases A and B*

The activities are expressed as a percentage of that at pH4.5 and 4.8. Citrate/phosphate buffer (0.1M-citric acid plus 0.2M-Na_2HPO_4) was used to adjust the pH to the desired value. ●, Hexosaminidase A; ○, hexosaminidase B.

polyacrylamide-gel disc electrophoresis. Although hexosaminidases A and B have been known to be present in various tissues and organisms for many years, these enzymes were never obtained in homogeneous forms from human kidney. The presence of other forms of hexosaminidases was previously shown by Sandhoff (1969). Price & Dance (1972) have detected two hexosaminidases in human serum, which they called I_1 and I_2. These forms appear to exhibit chromatographic behaviour intermediate between that of hexosaminidases A and B. Another form, hexosaminidase P, was also found in human serum by Stirling (1971). Srivastava *et al.* (1974*a*,*b*) purified hexosaminidases A and B from human placenta and found that the two enzymes differ in isoelectric point and *C*-terminal amino acid. Our purified hexosaminidases A and B displayed no other lysosomal acid hydrolase activity such as β-D-mannosidase, α-D-mannosidase, β-D-fucosidase, α-L-fucosidase, α-D-glucosidase, β-D-glucuronidase or β-D-galactosidase activity and were stable when stored at −20°C in phosphate buffer, pH6.5.

We obtained mol.wts. of 111000 and 114000 by gel filtration for human kidney hexosaminidases A and B. Our values are in agreement with those for the human placenta enzymes reported by Srivastava *et al.* (1974*b*), which were obtained by the sedimentation equilibrium technique, but are lower than those reported for the human placenta enzymes purified by Tallman *et al.* (1974) and Srivastava *et al.* (1974*b*), which were determined by gel filtration. Our molecular-weight determinations are in agreement with the values reported for the ox and human spleen enzymes as reported by Verpoorte (1972) and Robinson & Stirling (1968).

Since Carroll & Robinson (1973) and Tallman *et al.* (1974) have shown that both enzymes are glycoproteins, no definite conclusion can be drawn about

Table 2. *Effect of various activators and inhibitors on human kidney hexosaminidases A and B*

The enzymes were dialysed against water and assays for enzymic activity were performed as described in the text. The enzymes were incubated with the activator or inhibitor for 30min addition of 0.1 ml of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide. Results are expressed as the percentage of activity in the absence of added materials.

Compound	Concn. (mM)	Activity (%) A	B
Iodoacetic acid	1	108	112
Zn^{2+}	1	118	115
EDTA	1	111	112
Fe^{3+}	1	86	91
Cd^{2+}	1	96	84
Co^{2+}	1	100	96
Na^{+}	1	100	100
Ag^{+}	0.01	71	70
	1	0	0
Mg^{2+}	1	100	87
Hg^{2+}	0.0001	88	90
	1	0	0
p-Chloromercuri-benzoate	0.001	82	88
	1*	0	0
2-Mercaptoethanol	1	104	107
Sodium dodecyl sulphate	0.34	61	69
	0.68	7	10
	1	5	6
Urea	2.5M	42	37
	4.0M	31	25
	5.0M	21	11
	6.0M	8	5
	7.5M†	0	0
Heart albumin	10μl of 0.1%	150	130
	10μl of 0.1%‡	215	242

* Inhibition by $HgCl_2$ and *p*-chloromercuribenzoate was reversible. The enzyme activity could be restored to its initial values by the addition of 1 mM-cysteine.

† The enzymes were treated with 8M-urea. After the removal of urea by dialysis in the cold-room against 0.01M-Tris/HCl buffer, pH7.4, no enzyme activity could be detected.

‡ 4-Methylumbelliferyl *N*-acetyl-D-glucosaminide was used to determine enzyme activity.

the actual molecular weights of these enzymes, in view of the documented abnormal behaviour of glycoproteins on Sephadex-gel filtration (Andrews, 1965). When the molecular weights of the hexosaminidases A and B were determined by gel electrophoresis in the presence of sodium dodecyl sulphate, reducing agent and urea at neutral pH, we obtained different values. Hexosaminidase A exhibited a strong band corresponding to mol.wt. 68000 and trace amounts of a band with mol.wt. 140000. Hexosaminidase B gave under the same conditions bands with molecular weights ranging from 37000 to 140000. Both enzymes exhibited an apparent maximum mol.wt. of 140000. However, we believe that this value is an artifact caused by aggregation subsequent to dialysis and freeze-drying. We were unable to dissociate the native enzymes to subunits of 18000 mol.wt. by heating at 37°C for 3h in 8M-urea and 1% each of sodium dodecyl sulphate and 2-mercaptoethanol. Carboxy[^{3}H]methylated hexosaminidase A dissociated into subunits of mol.wts. 100000, 65000 and 18000, and carboxy[^{3}H]methylated hexosaminidase B dissociated into subunits of mol.wts. 37000 and 19000. These values for hexosaminidase B are the same as those reported by Srivastava *et al.* (1974*b*), but the values for hexosaminidase A differed from theirs. Segrest & Jackson (1972) have reported that glycoproteins behave anomalously during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis when compared with standard proteins.

Since the molecular weights of the major subunits of carboxymethylated hexosaminidases A and B are about 18000 from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plates 2*a* and 2*b*) and since the molecular weights of the native enzyme determined by Sephadex-gel filtration are 111000–114000, it is tempting to speculate that the native enzymes consist of six of these subunits. However, it is not known whether or not the polypeptide bands of mol.wt. 100000 and 65000 (hexosaminidase A) and mol.wt. 37000 (hexosaminidase B) are part of the multimeric structure of these enzymes or simply represent incomplete dissociation. Before any definite statements can be made it will be necessary to prepare larger amounts of the purified enzymes and separate subunits and determine their *N*- and *C*-terminal amino acids. It has been proposed (Srivastava *et al.*, 1974*b*) that hexosaminidase A is a heteropolymer $(\alpha,\beta)_3$ and hexosaminidase B is a homopolymer $(\beta,\beta)_3$. Carroll & Robinson (1973) and Beutler & Kuhl (1975) have also suggested that hexosaminidase A and B share a common subunit. The possibility that the subunits are linked together by a carbohydrate moiety has not been ruled out.

Inhibition of both hexosaminidase A and B by Hg^{2+} and *p*-chloromercuribenzoate indicates the presence of reduced disulphide groups in the soluble enzymes. Addition of cysteine led to a moderate stimulation (Table 2), suggesting that some disulphide groups can still be reduced with concomitant increase in enzyme activity, and that cysteine was required to maintain the reduced nature of the disulphide groups. Our results are in agreement with those reported for the ox spleen isoenzymes by Verpoorte (1972). Tallman *et al.* (1974) observed during the purification of hexosaminidase A that if partially purified enzyme was stored at 5°C, a small amount of this enzyme seemed to undergo transformation to hexosaminidase B. Such

observations had been made by Robinson & Stirling (1968).

We have been using an artificial glucopyranoside substrate to determine the various kinetic parameters of both enzymes. Our determination of the hydrolysis of the artificial substrate indicates that both enzymes have pH optima of 4.5 and 4.8, the same as for a partially purified enzyme from bovine uterus (Coleman *et al.*, 1967) and from pig kidney (Wetmore & Verpoorte, 1972). Braidman *et al.* (1974) suggested that the human hexosaminidases with acid pH optima are lysosomal hydrolases, whereas hexosaminidase C is located in the microsomal fraction and performs an independent function. They also suggested that they are under separate genetic control.

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References

Andrews, P. (1965) *Biochem. J.* **96**, 595–606
Armstrong, J. McD., Myers, D. V., Verpoorte, J. A. & Edsall, J. R. (1966) *J. Biol. Chem.* **241**, 5137–5149
Beutler, E. & Kuhl, W. (1975) *Nature (London)* **258**, 262–264
Braidman, I., Carroll, M., Dance, N. & Robinson, D. (1974) *Biochem. J.* **143**, 295–301
Carroll, M. & Robinson, D. (1973) *Biochem. J.* **131**, 91–96
Carroll, M. & Robinson, D. (1974) *Biochem. J.* **137**, 217–222
Coleman, R. L., Scroggs, R. A. & Whittington, A. (1967) *Biochim. Biophys. Acta* **146**, 290–292
Dance, N., Price, R. G., Robinson, D. & Stirling, J. L. (1969) *Clin. Chim. Acta* **24**, 189–197
Dance, N., Price, R. G. & Robinson, D. (1970) *Biochim. Biophys. Acta* **222**, 662–664
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Kolodny, E. H., Brady, R. O. & Volk, B. B. W. (1969) *Biochem. Biophys. Res. Commun.* **37**, 526–531
Li, Y. T., Mazzotta, M. Y., Wan, C. C., Ozth, R. & Li, S. C. (1973) *J. Biol. Chem.* **248**, 7512–7515
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Marinkovic, D. V. & Marinkovic, J. N. (1976) *Biochem. J.* **155**, 217–223
McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183–186
Murphy, J. V. & Craig, L. (1972) *Clin. Chim. Acta* **42**, 267–272
O'Brien, J. S., Okada, S., Ho, M. W., Fillerup, D. L., Veath, M. D. & Adams, K. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 956–968
O'Donnell, I. J., Frangione, B. & Porter, R. R. (1970) *Biochem. J.* **116**, 261–268
Okada, S. & O'Brien, J. S. (1969) *Science* **165**, 698–700
Price, R. G. & Dance, N. (1972) *Biochim. Biophys. Acta* **271**, 145–153
Robinson, D. & Stirling, J. L. (1968) *Biochem. J.* **107**, 321–327
Sandhoff, K. (1969) *FEBS Lett.* **4**, 351–354
Sandhoff, K., Harzer, K., Wassle, W. & Jatzkewitz, H. (1971) *J. Neurochem.* **18**, 2469–2489
Segrest, J. P. & Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63
Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820
Srivastava, S. K. & Beutler, E. (1972) *Biochem. Biophys. Res. Commun.* **47**, 753–759
Srivastava, S. K., Yoshida, A., Awasthi, Y. C. & Beutler, E. (1974*a*) *J. Biol. Chem.* **249**, 2043–2048
Srivastava, S. K., Yoshida, A., Awasthi, Y. C. & Beutler, E. (1974*b*) *J. Biol. Chem.* **249**, 2049–2053
Stirling, J. L. (1971) *Biochem. J.* **123**, 11P
Tallman, J. T., Brady, R. O., Quirk, J. M., Villalba, M. & Gal, A. E. (1974) *J. Biol. Chem.* **249**, 3489–3499
Verpoorte, J. A. (1972) *J. Biol. Chem.* **247**, 4787–4793
Wetmore, S. J. & Verpoorte, J. A. (1972) *Can. J. Biochem.* **50**, 563–573
Young, E. P., Ellis, R. B., Lake, B. D. & Patrick, A. D. (1970) *FEBS Lett.* **9**, 1–4