The Multiple Forms and Kinetic Properties of the *N*-Acetyl- β -D-hexosaminidases from Colonic Tumours and Mucosa of Rats Treated with 1,2-Dimethylhydrazine

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The separation and purification of the *N*-acetyl- β -D-hexosaminidase activities from tumours induced by 1,2-dimethylhydrazine in the rat colon and from colonic mucosa of tumour-bearing animals are reported. Mucosa contained *N*-acetylhexosaminidases A and B, as well as a third form whose properties with regard to electrophoretic mobility and thermostability lay between those of A and B. Tumours contained only *N*-acetylhexosaminidase A and B activities. Each form possessed both *N*-acetylglucosaminidase (EC 3.2.1.30) and *N*-acetylgalactosaminidase (EC 3.2.1.53) activities, which could not be separated by a variety of techniques. The alteration of the ratio of the two specific activities in each form during purification, together with differences in the kinetic inhibition constants and behaviour during inactivation by various reagents or a temperature of 50°C, supported the belief that each form contains the two enzyme activities, glucosaminidase and galactosaminidase, at separate active sites. This model is in contrast with that reported for these activities from a number of other sources. A variety of treatments reported to cause the conversion of form A into a form resembling B failed to produce such an effect on the rat colonic hexosaminidases.

The *N*-acetyl- β -D-hexosaminidases (2-acetamido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30, and 2-acetamido-2-deoxy- β -D-galactoside acetamidodeoxygalactohydrolase, EC 3.2.1.53) from a variety of sources exist in two major forms designated A and B. These forms have been reported to be similar with respect to molecular weight, pH optima, K_m values and behaviour towards inhibitors, but to differ in thermostability properties and electrophoretic mobility (Robinson, 1974). Various hypotheses have been put forward for a relationship between the two forms (e.g. Khawaja & Sellinger, 1976; Marinkovic & Marinkovic, 1977).

The enzymes have in general been reported as catalysing the release of *N*-acetylglucosamine and *N*-acetylgalactosamine from β -glycoside linkages at comparable rates, but the two activities have not been separated. However, many of the studies have measured only the glucosaminidase activity, but, where both activities have been compared kinetically, it appears that a common active site for the two types of glycoside is present (Woollen *et al.*, 1961; Walker *et al.*, 1961; Li & Li, 1970; Verpoorte, 1972; Aruna & Basu, 1975; Edwards *et al.*, 1975; Bouquelet & Spik, 1976).

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The hexosaminidase forms purified from rat colonic mucosa differ in kinetic properties and response to inactivation in a way that suggests separate active sites for the two types of glycoside substrate, each of which is inhibited by the substrate for the other (Mian *et al.*, 1978).

The present paper reports the extension of this work to the hexosaminidases of rat colonic tumours induced by 1,2-dimethylhydrazine treatment and compares their properties with those of the enzymes from the surrounding mucosa. We have previously shown that the tumour enzymes have higher specific activities (Mian & Cowen, 1974; Mian *et al.*, 1974) than, and differ significantly in kinetic properties (Mian *et al.*, 1975) from, the enzymes of normal colonic mucosa, with an absence of the intermediate form from tumours (Mian & Nutman, 1975).

Experimental

Colonic tumours were induced in male Wistar rats by 1,2-dimethylhydrazine, and the surrounding mucosa and tumours were collected separately as described by us previously (Mian *et al.*, 1975). In a histological survey of 25 colonic tumours, all were found to be well-differentiated adenocarcinomata, the majority being of the polypoid tubular type and showing little or no staining with *p*-aminosalicylate or Alcian Blue (Mian *et al.*, 1974).

Chromatographic materials, neuraminidase and thimerosal [ethyl-(2-mercaptobenzoato-S)-mercury, also known as thiomersal and Merthiolate] were obtained from the sources noted by Mian *et al.* (1978), and other reagents were of analytical grade. Cellogel was purchased from Whatman Lab Sales Ltd., Maidstone, Kent, U.K.

Glycosidase assays

The activities of *N*-acetyl- β -D-glucosaminidase and *N*-acetyl- β -D-galactosaminidase were measured at pH 4.2 with *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide and *p*-nitrophenyl *N*-acetyl- β -D-galactosaminide [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] respectively as substrates, by incubation at 37°C for 1 h as described previously (Mian *et al.*, 1975). In some experiments where phenyl *N*-acetyl- β -D-glucosaminide (Sigma) was used as a substrate, the liberated phenol was measured as described by Stolbach *et al.* (1958). The enzyme activities were calculated as nmol of *p*-nitrophenol or phenol released/h per mg of protein. Protein concentration was routinely measured by the biuret, Lowry or u.v.-absorption-ratio method (Layne, 1957), with crystallized bovine albumin (BDH Chemicals, Poole, Dorset, U.K.) as standard.

Electrophoresis

Cellogel electrophoresis was carried out by the method of Poenaru & Dreyfus (1973) by using 0.04M-potassium phosphate buffer, pH 6.6, for 1.5 h at 4°C and 200 V (potential gradient 13.3 V·cm⁻¹). Polyacrylamide-gel electrophoresis was carried out as described by Davis (1964). Strips and gels were stained for protein with Amido Schwartz and for enzyme activity with histochemical substrates (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) as described by Hayashi (1965).

Kinetic studies

In enzyme kinetic experiments, substrate concentrations in the final assay mixture ranged from 0.2 to 1.25 mM. In inhibition studies, portions of enzyme solutions were mixed with different concentrations of the inhibitors and incubated at 37°C for 15 min before the addition of the substrate solution. Freshly prepared solutions of *N*-acetylglucosaminolactone (2-acetamido-2-deoxy-D-gluconolactone) and the corresponding galactose analogue (Koch-Light) were heated at pH 2 by the method of Levvy *et al.* (1962) and diluted and adjusted to pH 4.2 immediately before use as inhibitors. Other sugar inhibitors were purchased from Sigma.

Analysis of the kinetic data was carried out as described by Mian *et al.* (1975) to give the statistically

best values of Michaelis constants (K_m), maximum velocities (V) and inhibition constants (K_i). Parameter values for enzymes from different tissues were examined by Student's *t* test to determine whether there was a significant difference.

Results

Purification of *N*-acetylhexosaminidases

Colonic tumours or mucosa were homogenized in 0.01M-KH₂PO₄/Na₂HPO₄ (pH 6.0)/0.1M-NaCl for 5 min at 4°C, followed by centrifugation at 40000g for 30 min. The supernatants were passed through a Sepharose 6B column (100cm×2.5cm diam.), equilibrated and eluted with the same buffer. The fractions containing enzyme activity were pooled, freeze-dried and dialysed for 16 h at 4°C against the above buffer and applied to a Sephadex G-200 column (100cm×2.5cm diam.). The active effluent fractions were concentrated and desalted by dialysis for 16 h at 4°C against 0.01M-KH₂PO₄/Na₂HPO₄, pH 6.0, and applied to a DEAE-cellulose column (50cm×2cm diam.) in the same buffer. The results of washing with this buffer and then introducing an increasing [NaCl] gradient are shown in Figs. 1 and 2. This procedure resulted in the separation of the mucosal enzyme activity into three forms (Fig. 1) and the tumour activity into two forms (Fig. 2). The enzyme eluted in the void volume was designated form B and the forms eluted by increasing concentrations of NaCl were designated I and A respectively. The terminology A and B follows that proposed by Robinson & Stirling (1968), but the terminology I for the intermediate form in the present study is arbitrary and bears no relation to the forms I₁ and I₂ of normal human serum *N*-acetyl- β -D-hexosaminidase that have been described by Price & Dance (1972). Form I was absent from tumour preparations.

The different forms were then individually purified according to the scheme given in Table 1. The activities of both *N*-acetylglucosaminidase and *N*-acetyl-galactosaminidase were measured in all fractions at each stage. Purification data in terms of *N*-acetylglucosaminidase activity are summarized in Table 2 for each enzyme form after the final stage in Table 1. Yields were not important, as the emphasis was on the relative behaviour of the two hexosaminidase activities.

Polyacrylamide-gel electrophoresis of each of the purified forms showed a single protein band containing both enzyme activities. The mobilities of the enzyme forms from cathode to anode were in the order B<I<A, similar to the elution sequence from DEAE-cellulose.

Cellogel electrophoresis of samples of supernatant failed to detect an enzyme form more anodic than the purified form A of *N*-acetylhexosaminidase. Chroma-

Fig. 1. DEAE-cellulose chromatography of colonic mucosal *N*-acetylhexosaminidase

The supernatant from homogenized mucosa was passed through columns of Sepharose 6B and Sephadex G-200, concentrated and dialysed against 0.01 M-KH₂PO₄/Na₂HPO₄, pH 6.0. The sample was applied to a column (50 cm × 2 cm diam.) of Whatman DEAE-cellulose DE-52, washed with 110 ml of the same buffer, followed by 220 ml of a linear increasing concentration (0–0.3 M) of NaCl in the same buffer. Fraction volumes were 3.5 ml. In each fraction the *N*-acetylglucosaminidase activity (●), *N*-acetylgalactosaminidase activity (○), *A*₂₈₀ (■) and, where applicable, the [NaCl] (—) were measured, the last by conductivity.

Fig. 2. DEAE-cellulose chromatography of *N*-acetylhexosaminidase from colonic tumours

Details are as for the mucosal enzyme in Fig. 1. The activities of *N*-acetylglucosaminidase from fractions 16–48 and of *N*-acetylgalactosaminidase from fractions 14–58 were zero, and the points are not shown for clarity.

tography on a column (100 cm × 2.5 cm diam.) of Bio-Gel P-200, prepared as described by Overdijk *et al.* (1975), similarly failed to reveal an additional enzyme-activity peak near the void volume when the effluent fractions were assayed at pH 5.5, 6.0 and 7.5. None of the fractions would preferentially hydrolyse one or other of the substrates, *p*-nitrophenyl *N*-acetylglucosaminide or *p*-nitrophenyl *N*-acetylgalactosaminide, when assayed at any of the above pH values. The only enzyme-containing peak found in the effluent profiles was a decreased version of the peak routinely observed when the enzyme activity was assayed at pH 4.2.

Incubation of each of the purified enzyme preparations with the appropriate 1 mM-*p*-nitrophenyl glyco-

side in 62.5 mM-sodium citrate buffer, pH 4.2 or 5.0, at 37°C for 1 h failed to detect any of the following enzyme activities at a level greater than 0.02% of the specific activity of *N*-acetylglucosaminidase: α -D-glucosidase, β -D-glucosidase, α -D-galactosidase, β -D-galactosidase, α -D-mannosidase, β -D-mannosidase, α -L-fucosidase, β -D-fucosidase and β -D-glucuronidase.

pH optimum

Citrate/phosphate and glycine buffers were used to cover the pH range 2.5–10 (Gomori, 1955). Both *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities showed pH optima at 4.2, in agree-

Table 1. *Experimental conditions for the further purification of N-acetylhexosaminidase by chromatographic steps*

The pooled active fractions from the first DEAE-cellulose separation were treated as shown. After each step, the pooled fractions were concentrated and dialysed against the buffer for the next step. For gradient elution steps, a preliminary wash of the volume noted, after application of the sample, was followed by an increasing [NaCl] gradient of volume and range as noted. Buffer systems used were as follows: a, 0.01 M-KH₂PO₄/Na₂HPO₄, pH 6.0; b, 0.01 M-KH₂PO₄/Na₂HPO₄, pH 5.0; c, 0.025 M-KH₂PO₄/Na₂HPO₄, pH 6.0; d, 0.02 M-sodium acetate, pH 5.0; e, 0.1 M-KH₂PO₄/Na₂HPO₄, pH 7.5; f, 0.01 M-KH₂PO₄/Na₂HPO₄, pH 7.5.

Chromatographic step	Bed height × diam. (cm)	Buffer system	Volume of wash (ml)	Volume of gradient (ml)	NaCl gradient range (M)
For <i>N</i> -acetylhexosaminidase A and I					
Second DEAE-cellulose (Whatman DE-52)	50×2	a	110	220	0–0.3
DEAE-Sephadex A-25	30×2	c	150	300	0–0.6
CM-Sephadex C-25	20×0.9	d	100	250	0–0.5
ECTEOLA-cellulose	40×0.9	f	0	250	0–0.2
Concanavalin A–Sephrose	5×0.25	e	35	Eluted with 1.0M- α -methyl glucoside Eluted with 0.1 M-NaCl	
Sephadex G-200	40×1.5	b	0		
For <i>N</i> -acetylhexosaminidase B					
Second DEAE-cellulose (Whatman DE-52)	50×2	a		No gradient	
CM-cellulose (Whatman CM-52)	15×0.9	b	70	250	0–0.4
DEAE-Sephadex A-25	30×2	c		No gradient	
CM-Sephadex C-25	20×0.9	d	250	250	0–0.5
Concanavalin A–Sephrose	5×0.25	e	35	Eluted with 1.0M- α -methyl glucoside Eluted with 0.1 M-NaCl	
Sephadex G-200	40×1.5	b	0		

Table 2. *Summary of the purification data for the three forms of N-acetylhexosaminidase from colonic mucosa and tumours*
Data are listed for the supernatant from the tissue homogenate and for each purified form after the last stage in Table 1.

Enzyme preparation	Total protein (mg)	N-Acetylglucosaminidase activity			Yield (%)	Specific-activity ratio of glucosaminidase to galactosaminidase
		Total activity (μmol/h)	Specific activity (nmol/h per mg of protein)	Purification factor		
Colonic mucosa of tumour-bearing rats						
Supernatant	6580	69.8	11	1	100	1.2
Form A	0.13	6.7	52800	4800	9.6	5.6
Form B	0.10	5.9	56200	5110	8.4	3.6
Form I	0.04	1.7	45100	4100	2.4	5.6
Colonic tumours						
Supernatant	5560	118.0	21	1	100	1.5
Form A	0.10	11.1	107000	5090	9.4	5.0
Form B	0.09	11.0	118000	5620	9.3	2.2

ment with the results reported for such enzymes for normal rat colonic mucosa (Mian *et al.*, 1978).

K_m and V values

Table 3 lists the values of the kinetic parameters for the hydrolysis of each substrate by these colonic

enzymes. In every case, the values of K_m and V were considerably greater when the glucosaminide substrate was employed. For each substrate, the K_m values of each form were significantly different ($P < 0.005$ to $P < 0.001$) in the sequence of A > I > B. No such differences were noted for the V values. The K_m values for form B of the tumour enzymes were

significantly lower ($P < 0.001$) than the values for form B in the colonic mucosa of tumour-bearing animals, whereas there were no such differences for form A.

Kinetics of inhibition by substrate analogues

Monosaccharides such as D-glucose, D-galactose, D-glucosamine, D-galactosamine, D-mannose and *N*-acetylmannosamine had no significant effect on the activities of the hexosaminidases. However, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-

glucosaminolactone, *N*-acetylgalactosaminolactone and D-mannosamine were competitive inhibitors with respect to both *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide and *p*-nitrophenyl *N*-acetyl- β -D-galactosaminide. The results are given as K_i values in Table 4. The *N*-acetylhexosaminolactones were the most effective of the inhibitors, and in each pair of analogues the galactosyl member inhibited more strongly than the glucosyl member. Each compound inhibited the galactosaminidase activity less strongly than the glucosaminidase activity, although not every instance was statistically significant. In general, the

Table 3. *Michaelis constants and maximum velocities for the N-acetylhexosaminidases*
Values are the means $\pm$ S.E.M. for 20 experiments.

Enzyme	<i>p</i> -Nitrophenyl <i>N</i> -acetylglucosaminide		<i>p</i> -Nitrophenyl <i>N</i> -acetylgalactosaminide	
	K_m (mM)	V (nmol of product/h per mg of protein)	K_m (mM)	V (nmol of product/h per mg of protein)
Colonic mucosa				
A	0.76 ± 0.18	0.74 ± 0.18	0.33 ± 0.02	0.092 ± 0.023
B	0.31 ± 0.08	0.69 ± 0.22	0.095 ± 0.035	0.076 ± 0.022
I	0.53 ± 0.12	0.62 ± 0.10	0.20 ± 0.04	0.047 ± 0.013
Colonic tumours				
A	0.73 ± 0.13	0.76 ± 0.12	0.33 ± 0.09	0.078 ± 0.016
B	0.26 ± 0.04	0.68 ± 0.17	0.064 ± 0.018	0.062 ± 0.010

Table 4. *K_i values of competitive inhibitors of the N-acetylhexosaminidases*

Values are the means $\pm$ S.E.M. for ten experiments. The values in column 5 are significantly lower ($P < 0.001$) than the corresponding values in column 4. The values in column 3 are significantly lower ($P < 0.001$) than those in column 1 and significantly higher ($P < 0.001$) than those in column 2.

Enzyme form	K_i				
	1 <i>N</i> -Acetyl- glucosamine (mM)	2 <i>N</i> -Acetyl- galactosamine (mM)	3 Mannosamine (mM)	4 <i>N</i> -Acetyl- glucosamino- lactone (μ M)	5 <i>N</i> -Acetyl- galactosamino- lactone (μ M)
Colonic mucosal enzyme					
<i>p</i> -Nitrophenyl <i>N</i> -acetylglucosaminide as substrate					
A	5.9 ± 1.0	0.56 ± 0.10	3.2 ± 0.7	5.0 ± 1.1	2.4 ± 0.7
B	4.2 ± 0.6	0.36 ± 0.07	1.8 ± 0.4	3.9 ± 0.9	1.9 ± 0.6
I	4.9 ± 0.9	0.42 ± 0.09	2.3 ± 0.4	4.4 ± 0.9	2.2 ± 0.8
<i>p</i> -Nitrophenyl <i>N</i> -acetylgalactosaminide as substrate					
A	14.6 ± 4.4	2.1 ± 0.6	6.4 ± 0.9	12.0 ± 3.7	8.1 ± 1.4
B	4.8 ± 0.9	1.5 ± 0.1	2.9 ± 0.5	4.6 ± 0.8	2.0 ± 0.5
I	7.7 ± 1.9	1.9 ± 0.1	4.8 ± 0.9	9.5 ± 2.1	2.9 ± 0.7
Colonic tumour enzyme					
<i>p</i> -Nitrophenyl <i>N</i> -acetylglucosaminide as substrate					
A	5.4 ± 1.1	0.59 ± 0.13	2.9 ± 0.5	4.3 ± 1.1	3.0 ± 0.5
B	2.5 ± 0.5	0.28 ± 0.07	1.3 ± 0.4	1.2 ± 0.1	0.72 ± 0.07
<i>p</i> -Nitrophenyl <i>N</i> -acetylgalactosaminide as substrate					
A	12.8 ± 2.5	2.9 ± 0.6	6.4 ± 0.6	9.7 ± 1.5	7.9 ± 1.0
B	2.9 ± 0.3	0.96 ± 0.05	1.7 ± 0.4	1.9 ± 0.1	1.01 ± 0.04

three enzyme forms were inhibited by a particular compound in the order $A < I < B$. It should be noted that the B forms of both tumour enzyme activities were significantly more strongly inhibited ($P < 0.005$) by all five compounds than the B forms of the enzymes from the colonic mucosa of tumour-bearing rats (Table 4).

Effects of metal ions, EDTA, arsenite, iodoacetate, N-ethylmaleimide and dithioerythritol

Treatment of each of the enzyme forms from colonic mucosa with 1–5 mM solutions of various metal chlorides (Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+}) or of sodium EDTA, sodium arsenite, sodium iodoacetate or *N*-ethylmaleimide, for 30 min at 37°C, had no significant effect on either activity, measured after a 25-fold dilution. Preincubation with 2.5 mM-dithioerythritol, however, decreased both activities of all three forms by about 20% at pH 4.2 and 25% at pH 7.0 (assayed after 25-fold dilution at pH 4.2). Similar observations were also made with the enzymes from colonic tumours.

Effect of $AgNO_3$ and $HgCl_2$

Each enzyme form from colonic mucosa was incubated with $AgNO_3$ or $HgCl_2$ for various time intervals at 37°C and pH 4.2, and the remaining glucosaminidase activity then determined in the usual

manner after dilution. The effect of time of treatment on activity remaining is shown in Fig. 3. $HgCl_2$ was the more rapid inactivator and the inactivation of all three forms of the enzyme by both compounds was a simple first-order decay process (Fig. 3). Similar experiments at pH 5.6 and 7.0 indicated that enzyme inactivation by $HgCl_2$ and $AgNO_3$ was independent of pH. The rates of inactivation of the different forms were in the order $I > B > A$ for $AgNO_3$ and $A > I > B$ for $HgCl_2$ (Fig. 3). The same pattern was found when the *N*-acetylgalactosaminidase activity was measured.

Effect of thimerosal

The results of similar experiments on the effect of the time of treatment with 1 mM-thimerosal on the remaining glucosaminidase activity in both mucosal and tumour enzymes are shown in Fig. 4. Thimerosal at pH 4.2 and 37°C caused a biphasic decay of the two enzyme activities, comprising a rapid inactivation in the first 60 min followed by a slower effect over a period of several hours. The effect was independent of pH over the range 4.2–7.0. Separate experiments in which the thimerosal concentration was varied showed that the concentrations needed to decrease the original activity by 50% after treatment for 1 h at 37°C were 0.20 mM for form A, 0.65 mM for form I and 1.75 mM for form B (Fig. 5). No difference in the response of the two enzymic activities was noted.

Fig. 3. Time-dependent inactivation of purified colonic mucosal *N*-acetylglucosaminidase by $HgCl_2$ and $AgNO_3$. Samples were treated for various times with 10 μM - $AgNO_3$ (all forms), 1.5 μM - $HgCl_2$ (forms A and I) or 5 μM - $HgCl_2$ (form B) at pH 4.2 and 37°C. The remaining *N*-acetylglucosaminidase activity was measured after 25-fold dilution as a function of treatment time, and expressed as a percentage of the activity found in controls in the absence of added heavy metals. Each point is the average of three experiments. (a) $AgNO_3$; (b) $HgCl_2$. ●, Form A; ▲, form B; ▼, form I.

Fig. 4. Time-dependent inactivation of purified colonic mucosal and tumour *N*-acetylglucosaminidase by thimerosal. Samples were treated for various times with 1 mM-thimerosal in 62.5 mM-citrate buffer, pH 4.2, at 37°C. The remaining *N*-acetylglucosaminidase activity was measured after 25-fold dilution as a function of treatment time, and expressed as a percentage of the activity found in controls in the absence of thimerosal. Each point is the average of three experiments. (a) Colonic mucosal enzyme; (b) tumour enzyme. ●, Form A; ▲, form B; ▼, form I.

Fig. 5. Effect of thimerosal concentration on purified colonic mucosal *N*-acetylglucosaminidase.

Colonic mucosal enzyme samples were incubated with various concentrations of thimerosal in 62.5 mM-citrate buffer, pH 4.2, at 37°C for 1 h. The enzyme activity remaining was determined in the usual manner after a 25-fold dilution and expressed as a percentage of the activity found in controls treated under similar conditions without thimerosal. Each point is the average of three experiments. ●, Form A; ▲, form B; ▼, form I.

Effect of neuraminidase

Enzyme samples were treated at 37°C for 18 h with 100 units of neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* or *Vibrio cholerae* in 50 mM-

sodium phosphate/sodium citrate buffer, pH 5.6, containing 20 mM- CaCl_2 . Measurement of the two hexosaminidase activities at pH 4.2 did not show more than a 10% decrease, a similar loss being found in controls incubated in the absence of neuraminidase. The different forms from the two colonic tissues showed no difference in response.

Effect of *p*-hydroxymercuribenzoate

The effect of *p*-hydroxymercuribenzoate was dependent on pH and on the form of the enzyme being inactivated. The activities of forms A and I of the glucosaminidase from colonic mucosa were decreased by 50% or more after treatment with 5 μM -*p*-hydroxymercuribenzoate at pH 4.2, 5.6 and 7.0 for 15 min at 37°C, whereas form B was stable at pH 4.2 and 7.0 and inactivated by less than 5% at pH 5.6. A similar pattern was found for the galactosaminidase activities of the three enzyme forms, although a much higher concentration of *p*-hydroxymercuribenzoate (100 μM) was needed to decrease the activity of forms A and I by 50%, whereas form B was stable at all three pH values. A study of the inactivation as a function of time indicated that both activities of form B were inactivated more slowly than those of the other forms (Fig. 6). Among the enzyme activities from tumours, the B form was slightly more resistant to inactivation by *p*-hydroxymercuribenzoate than was the B form from mucosa (Fig. 7).

Protection against inactivation

Addition of glutathione (100 mM) or L-cysteine (10 mM) before or after treatment of enzyme samples

Fig. 6. Time-dependent inactivation of purified colonic mucosal *N*-acetylhexosaminidase by *p*-hydroxymercuribenzoate. Samples were treated for various times with (a-c) 10 μ M- or (d-f) 200 μ M-*p*-hydroxymercuribenzoate at pH 4.2, 5.6 or 7.0 and 37°C. The remaining enzyme activities were measured as a function of treatment time after a 25-fold dilution and adjustment of the pH to 4.2, and expressed as a percentage of the activity found in controls in the absence of *p*-hydroxymercuribenzoate. Each point is the average of three experiments. (a-c) *N*-Acetylglucosaminidase activity; (d-f) *N*-acetylgalactosaminidase activity. (a) and (d) pH 4.2; (b) and (e) pH 5.6; (c) and (f) pH 7.0. ●, Form A; ▲, form B; ▼, form I.

with HgCl_2 , AgNO_3 , thimerosal or *p*-hydroxymercuribenzoate did not prevent or reverse the inactivation. Similarly the presence of dithioerythritol (2.5 mM) before inactivation by thimerosal or *p*-hydroxymercuribenzoate, or its addition afterwards with adjustment of the pH to 7.0 (Beutler *et al.*, 1976), failed to maintain or restore enzyme activity. On the other hand, although the presence of the respective substrates during inactivation by HgCl_2 , AgNO_3 or thimerosal did not affect the loss of the two enzyme activities, their presence did decrease the extent of the inactivation by *p*-hydroxymercuribenzoate. The presence of *p*-nitrophenyl *N*-acetylglucosaminide at a concentration 50 times that of the inactivator decreased the rate of loss of glucosaminidase activity by 20.5%, whereas the presence of the corresponding galactosaminide substrate increased the inactivation rate of the glucosaminidase activity, measured with phenyl *N*-acetylglucosaminide as substrate. On the other hand, the presence of *p*-nitrophenyl *N*-acetylgalactos-

aminide at a concentration 10 times that of *p*-hydroxymercuribenzoate lowered the rate of inactivation of the galactosaminidase activity by 25.5%, whereas there was no effect from phenyl *N*-acetylglucosaminide, a substrate for the other activity.

The B form of the tumour activities again behaved differently from the B form of the mucosal enzymes. It was protected to a greater degree by a lower concentration of substrate; for example, the rate of inactivation of the glucosaminidase activity was lowered by 40.5% by the glucosaminide substrate at a concentration 15 times that of *p*-hydroxymercuribenzoate, the corresponding values for the galactosaminidase activity and the galactosaminide substrate being 55.5% at 5 times.

Thermal inactivation

A rapid and almost complete inactivation of the three forms was observed on heating the mucosal

Fig. 7. Time-dependent inactivation of purified colonic tumour *N*-acetylhexosaminidase by *p*-hydroxymercuribenzoate. Details of the treatment are as in Fig. 6. Each point is the average of three experiments. (a), (b), (c) *N*-Acetylglucosaminidase activity; (d), (e), (f) *N*-Acetylgalactosaminidase activity. (a) and (d) pH 4.2; (b) and (e) pH 5.6; (c) and (f) pH 7.0. ●, Form A; ▲, form B.

enzyme samples for 5 min at 55°C or 60°C. However, at 50°C, the inactivation was slower and for both activities the rates of inactivation were in the order $B < I < A$ (Fig. 8). Both activities of the tumour B form showed greater stability to thermal inactivation at 50°C than the B form of the mucosal enzymes (Fig. 8).

Non-interconvertibility of different forms of the hexosaminidases

The purified enzyme forms were subjected to various treatments reported to bring about interconversion, but no evidence could be found for forms A or I being transformed to a form resembling B. Phosphate/citrate buffers (50 mM) were prepared as described by Gomori (1955), and treatment included (a) 100 units of neuraminidase in phosphate/citrate (pH 5.6)/20 mM-CaCl₂, for 18 h at 37°C; (b) 1 mM-thimerosal in phosphate/citrate, pH 4.2, 5.6 and 7.0 for 1 h and 18 h at 37°C; (c) 1.5 μ M-HgCl₂ or 10 μ M-AgNO₃ in phosphate/citrate, pH 4.2 and 7.0 for 1 h at 37°C; (d) 5 μ M-*p*-hydroxymercuribenzoate in phosphate/citrate, pH 4.2, 5.6 and 7.0 for 1 h and 18 h at 37°C; (e) heating for 15 min at 50°C; (f) repeated freezing and thawing in 3 M-NaCl; (g) repeated chromatography on DEAE-cellulose at pH 7.0.

None of these treatments resulted in the conversion of forms A or I into a form resembling B. DEAE-

cellulose chromatography after treatment failed to detect any enzyme activity or protein corresponding to form B, nor was greater stability to thermal inactivation produced. Kinetic studies showed that treatment with thimerosal or neuraminidase did not significantly alter the K_m and K_i values for the substrates and inhibitors described above from those expected for A and I forms.

Discussion

The separation of the *N*-acetylhexosaminidase activities in the colonic mucosa of tumour-bearing rats into three forms (form A, form B and an intermediate form we have called form I) was exactly as expected from our results with normal rat mucosa (Mian *et al.*, 1978), but colonic tumours were found to lack the intermediate form (Fig. 2). This kind of modification of isoenzyme pattern has been observed in many cases of malignant transformation (Schapira, 1973), particularly for hexosaminidase isoenzymes in rat hepatomas (Weber *et al.*, 1973).

The intermediate form, arbitrarily termed I, described in this paper and elsewhere (Mian *et al.*, 1978), had properties intermediate between those of hexosaminidases A and B with respect to electrophoretic mobility, elution position from DEAE-cellulose and thermostability, but did not correspond to forms I₁ and I₂ of normal human serum *N*-acetyl-

Fig. 8. Thermal inactivation of *N*-acetylhexosaminidase at 50°C

Enzyme samples in 62.5 mM-citrate buffer, pH 4.2, were heated at 50°C for different time intervals and cooled to 4°C before assay under the usual conditions. The results are the averages of three experiments and are expressed as a percentage of the activity of controls kept at 4°C. (a) and (b) *N*-Acetylglucosaminidase; (c) and (d) *N*-Acetylgalactosaminidase. (a) and (c) Mucosa; (b) and (d) tumour. ●, Form A; ▲, form B; ▼, form I.

β -D-glucosaminidase, which have been reported to be thermostable at 50°C (Price & Dance, 1972).

Failure, by procedures of Braidman *et al.* (1974*a,b*), Overdijk *et al.* (1975) and Besley & Broadhead (1976), to detect an enzyme form more anodic than hexosaminidase A on Cellogel electrophoresis (Poenu & Dreyfus, 1973) or any additional activity peak at pH 5.5, 6.0 or 7.5 near the void volume after chromatography on Bio-Gel P-200 (Overdijk *et al.*, 1975) or a form that showed a preferential activity towards *p*-nitrophenyl *N*-acetylglucosaminide or *p*-nitrophenyl *N*-acetylgalactosaminide substrates (Overdijk *et al.*, 1975) implied that hexosaminidase C (Hooghwinkel *et al.*, 1972) was not present in the colonic tissues.

The degree of purification and percentage yields for the different forms of colonic *N*-acetylglucosaminidase in the present work (Table 2) are similar to our previous observations (Mian *et al.*, 1978) and to other reports (Verpoorte, 1972; Carroll & Robinson, 1973; Srivastava *et al.*, 1974; Tallman *et al.*,

1974; Aruna & Basu, 1975; Ellis *et al.*, 1975; Marinkovic & Marinkovic, 1977). The specific-activity ratios of glucosaminidase to galactosaminidase in the supernatants of colonic tissues were less than 2, as we found for tumour-free rats (Mian *et al.*, 1978), but other sources showed different ratios (Verpoorte, 1972; Carroll & Robinson, 1973; Ellis *et al.*, 1975). For purified forms A and I, the ratios of 5 or a little greater were in agreement with Verpoorte (1972) and Mian *et al.* (1978), but not with others (Carroll & Robinson, 1973; Ellis *et al.*, 1975). Unlike the A forms, the ratios for the B forms differed considerably between tumours and the surrounding mucosa. If the ratios increase during purification by a relative instability of the galactosaminidase activity, then these observations suggest that this activity of form B, especially in tumours, is more stable than that of the other forms when exposed to the purification procedures of Table 1.

The purified enzymes from tumours had considerably greater specific activities than those from

the surrounding mucosa (Table 2), in agreement with our earlier findings for unpurified tissue homogenates (Mian & Cowen, 1974; Mian *et al.*, 1974). In general, form A of the tumour enzymes resembled form A of the mucosal enzymes, whether from normal (Mian *et al.*, 1978) or tumour-bearing rats, but certain differences were noted when comparing the B forms. The K_m and K_i values for the B form of the tumour activities were always lower than those for the corresponding mucosal activities (Tables 3 and 4). The similarity of the V values, on the other hand, indicates that K_m must be close to K_s , the dissociation constant of the enzyme-substrate complex, so that the effect of the neoplastic transformation, on the B forms at least, has been to increase the binding of substrates and competitive inhibitors to the active sites. The tumour B forms were also more stable under conditions of thermal inactivation, and, in agreement with the supposition of tighter substrate binding, were protected by substrate more effectively against inactivation by *p*-hydroxymercuribenzoate.

Cysteine, glutathione and dithiothreitol have been reported to reverse the inactivation by thiol-group reagents of hexosaminidases from other sources (Verpoorte, 1972; Beutler *et al.*, 1975; Marinkovic & Marinkovic, 1977), but no such effect was found for rat colonic enzymes (where dithioerythritol replaced dithiothreitol). The inactivating effect of thimerosal and *p*-hydroxymercuribenzoate on the human placental enzymes has been postulated to be due to alkylation of subunits (Beutler *et al.*, 1975, 1976), and restoration of activity by dithiothreitol has been demonstrated (Beutler *et al.*, 1976). However, we found dithioerythritol to be without effect as a protecting agent and indeed to cause inactivation itself at pH 7.

The presence of substrates protected the particular activity against loss by *p*-hydroxymercuribenzoate but not by the other reagents. Such differences in properties compared with the hexosaminidases from other sources may be due to different siting of the SH groups in relation to the active centre or centres of the enzymes. Those sensitive to *p*-hydroxymercuribenzoate may be protected via a conformational change, initiated by substrate binding, which cannot, however, affect the interaction with the smaller Ag^+ and Hg^{2+} ions. Similar observations have been reported for the hexosaminidases in normal rat colonic mucosa (Mian *et al.*, 1978) and for other enzyme systems (Jocelyn, 1972).

It may be postulated from the biphasic nature of the time-dependence of the inactivation by thimerosal (Fig. 4) and *p*-hydroxymercuribenzoate (Fig. 6) that there are two classes of SH group necessary for activity, one of which reacts more rapidly than the other. The two reagents are differentiated by the lack of protection by substrate against inactivation with thimerosal on the one hand, and the anomalous

results with *p*-hydroxymercuribenzoate on the other. These differences may reflect differences in the modes of action of the two reagents.

The absence of evidence for the conversion of forms A or I into a form resembling B enhances the view that the rat colonic hexosaminidases are different from those of other sources. Robinson & Stirling (1968) reported conversion by removal of neuraminyl groups, and Tallman *et al.* (1974) by a presumed conformational change after heating at 50°C. Repeated freezing and thawing in 3M-NaCl (Beutler & Kuhl, 1975) or treatment with thiol reagents (Beutler *et al.*, 1975, 1976; Srivastava *et al.*, 1976) led to the proposal of an $\alpha_2\beta_2$ structure for form A and a β_4 structure for form B. Seyama & Yamakawa (1974) and Khawaja & Sellinger (1976) suggested dissociation of an A-B complex, based on observation of interconversion by incubation at 37°C and pH 6.0 or rechromatography on DEAE-cellulose at pH 7.0. It is to be presumed that, as none of these procedures affected the rat colonic forms A and B, their relationship must fall into yet another category.

Inhibition of the enzyme activities by the *N*-acetylhexosamines and their lactone analogues but not by glucose, galactose, mannose, glucosamine, galactosamine or *N*-acetylmannosamine indicates a specificity for the equatorial acetamido group at C-2 of the pyranose ring. However, the strength of the inhibition by mannosamine is of some interest, as the difference between this amino sugar and the other amino sugars that do not inhibit is in the position of the amino group at C-2 of the pyranose ring. It appears, therefore, that at pH 4.2 the positively charged axial amino group in mannosamine may attract a negatively charged group in the active site of the enzyme to form a non-productive complex, or may otherwise hinder access of the enzyme to the substrate.

The K_i values for the lactones are several orders of magnitude lower than the substrate K_m or product K_i values (Tables 3 and 4). This agrees with the results of Leaback (1968), who argued that such a high affinity is evidence for a half-chair conformation, similar to that of the lactone, in the transition state of the enzyme-substrate reaction.

Finally, the observations reported here support our conclusions for the enzymes from normal rat colonic mucosa (Mian *et al.*, 1978) that there are two types of active site, each specific for one of the hexosaminidase activities. The glucosaminidase and galactosaminidase activities could not be separated; in agreement with the experience of many other laboratories (Verpoorte, 1972; Tallman *et al.*, 1974; Srivastava *et al.*, 1974; Aruna & Basu, 1975; Edwards *et al.*, 1975; Kiyohara *et al.*, 1976), yet, on purification, the specific-activity ratio of the two activities altered (Table 2). Each of the five competitive inhibi-

tors listed (Table 4) had different K_i values with respect to the two substrates; a common active site would require identical K_i values for a given inhibitor. Furthermore, the experimental findings that treatment with *p*-hydroxymercuribenzoate caused a loss of glucosaminidase activity that was decreased by the presence of a glucose substrate but increased by a galactose substrate, and a loss of galactosaminidase activity that was decreased by the presence of a galactose substrate but unaffected by the glucose substrate, supports the model of two active sites. A common active site would have resulted in protection of both activities to the same degree by a given substrate. The present data thus confirm our model previously proposed for the hexosaminidases from normal colonic mucosa (Mian *et al.*, 1978).

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