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# THE MULTIPLE FORMS AND KINETIC DIFFERENCES OF RAT COLONIC β-*N*-ACETYLHEXOSAMINIDASES

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## Summary

Rat colonic β-*N*-acetylhexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) has been separated into three forms by DEAE-cellulose chromatography with an increasing salt gradient. It was not possible to separate the glucosaminidase activity from the galactosaminidase activity by a variety of chromatographic procedures, but the ratio of the two specific activities varied during purification. The pH optima were however identical, for both activities and all three forms. Kinetic measurements including inhibition by substrate analogues showed differences between the two activities as well as among the three forms. A common active site model was inconsistent with the results. Data from mixed substrate experiments were consistent with a model wherein the two activities reside in separate active sites, each able to be inhibited by the substrate for the other site. The effect of acetate and SH reagents confirmed the two-site model.

Treatment with neuraminidase, thimerosal, *p*-hydroxymercuribenzoate, $HgCl_2$ and $AgNO_3$ or heating at 50° C did not produce any effect on the A form that could be identified as a conversion to the B form. Measurement of the effects on both activities supported the two-site model. It is concluded that the relationship between the A and B forms in the rat colonic mucosa hexosaminidases must be different from that reported for such enzymes from other sources.

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## Introduction

Two major forms of β-*N*-acetylhexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) designated A and B can be separated from a variety of sources by electrophoretic and ion-exchange chromatographic procedures [1,2]. Preliminary studies on the enzyme from rat colonic mucosa showed the existence of a third form (form I) with electrophoretic mobility on polyacrylamide gels intermediate between those of the A and B forms [3].

The enzymes have in general been found to hydrolyse β-*N*-acetylglucosaminides and β-*N*-acetylgalactosaminides at comparable rates (for example, refs. 4—6) with the exception of some brain tissue enzymes where considerably greater specificity has been reported [7,8]. The two activities have proved to be inseparable by available fractionation procedures, leading to the suggestion that they are associated with the same enzyme protein. Enzyme kinetic methods have been applied to tissue extracts and purified enzymes (for example, refs. 4, 6 and 9) to investigate whether the two activities share a common active site, and in general the results are consistent with the proposal that both β-*N*-acetylhexosaminide substrates are indeed hydrolysed at the same active sites of the enzyme molecule. However, our kinetic data with the unpurified enzymes from rat colonic tissues, and particularly the results with reversible inhibitors, cannot be explained on the basis of a common active site for the two substrates [10].

The literature contains conflicting reports about the similarly of the kinetic properties of the A and B forms, and some authors believe that the different forms are related and are interconvertible. For example, Robinson and Stirling [1] explained the action of neuraminidase on form A to produce a form resembling B by proposing that form B was an aneuraminyl derivative of form A. While some reports from other laboratories confirmed this hypothesis [11,13], conversion of form A into a B-like form was later shown to be due to thimerosal (ethylmercurithiosalicylate) which is the preservative in commercial preparations of neuraminidase [14—16]. Furthermore, it was reported that other reagents for SH groups such as *p*-hydroxymercuribenzoate and $AgNO_3$ could bring about this conversion [16—18]. Beutler's group [19] also reported that form A could be converted directly to form B by repeated freezing and thawing in buffered 3 M NaCl. They proposed that form B consists of ββ subunits while form A is composed of αβ subunits.

Tallman and coworkers [5] reported that conversion of form A into a form resembling B could be achieved simply by heating at 50°C and suggested that the two forms were different conformational states of the same protein molecule.

The present work was undertaken to investigate the comparative behaviour of the two enzyme activities upon purification of the three forms of the rat colonic enzymes, and to study in detail the kinetics of each form in order to elucidate the relationship between the two activities. The effect of the various reagents that have been used by other investigators in their enzyme conversion studies was also of interest, to see what relationship between the A, B and I forms could be discovered.

## Materials and Methods

Colonic mucosa were collected from approx. 30-week-old male Wistar rats as described previously [20].

DEAE-cellulose (DE-52) and carboxymethylcellulose (CM-52) were purchased from Whatman, and ECTEOLA-cellulose was obtained from Sigma. Sephadex G-200, DEAE-Sephadex A-25, CM-Sephadex C-25, Sepharose 6B and Con A-Sepharose 4B were bought from Pharmacia.

Neuraminidases from *Clostridium perfringens* (Sigma, type VI) and from *Vibrio cholerae* (B.D.H. Chemicals and Koch-Light Laboratories) were used. Thimerosal was obtained from Sigma. Other reagents used were analytical reagent grade.

The activities of β-*N*-acetylglucosaminidase (EC 3.2.1.30) and β-*N*-acetylgalactosaminidase (EC 3.2.1.53) were measured at pH 4.2 using *p*-nitrophenyl-β-*N*-acetylglucosaminide and *p*-nitrophenyl-β-*N*-acetylgalactosaminide (Sigma), respectively, as substrates by the method described previously [10]. In some cases, phenyl-β-*N*-acetylglucosaminide (Sigma) was used, when phenol was estimated as described by Stolbach et al. [21]. The enzyme activities were calculated as nmol of *p*-nitrophenol or phenol released/h per mg protein.

In the routine kinetics experiments, substrate concentrations ranged from 0.2 to 1.25 mM. In the inhibition experiments, aliquots of enzyme solution were mixed with different concentrations of the inhibitors and incubated at 37° C (the temperature of the kinetic runs) for 15 min prior to the addition of the substrate solution. Analysis of the kinetic data was carried out as described by us previously [10], to give the statistically best values of Michaelis constants ($K_m$), maximum velocities ($V$) and inhibition constants ($K_i$). Student's $t$-test was used to determine whether two values were significantly different.

Incubation of enzyme samples with neuraminidase was carried out in 50 mM citrate/phosphate buffer (pH 5.6), 20 mM $CaCl_2$, at 37° C for different periods. At the end of the reaction, the pH was adjusted to 4.2 for assaying the enzyme activity. Reactions of the enzyme samples with other reagents were carried out at pH 4.2 unless otherwise mentioned. The treated enzyme samples were dialysed for 18 h against several changes of 0.1 M sodium/potassium phosphate buffer (pH 6.0) before chromatography on DEAE-cellulose, thermal inactivation at 50° C or their use in kinetics experiments.

## Results

*Purification of β-N-acetylhexosaminidases*

Colonic tissues were homogenised in 0.01 M sodium/potassium phosphate buffer (pH 6.0), 0.1 M in NaCl, for 5 min at 4° C, followed by centrifugation at 40 000 × $g$ for 30 min. The supernatants were collected and passed through a Sepharose 6B column, equilibrated and eluted with the same buffer. The fractions containing enzyme activity were rechromatographed on Sephadex G-200 in the same buffer, concentrated and desalted by dialysis against 0.01 M sodium/potassium phosphate buffer (pH 6.0) and applied to a column of DE-52 for elution with an increasing linear 0—0.3 M NaCl gradient. The hexosaminidases were separated into three different forms by this process. The

enzyme eluted in the void volume was designated hexosaminidase B, and the forms eluted by increasing concentrations of NaCl were designated I and A, respectively. The results of protein concentration and activity measurements for these steps are given in Table I in terms of β-*N*-acetylglucosaminidase activity.

Each form was then subjected to rechromatography on DE-52, CM-52 (form B only), DEAE-Sephadex A-25, CM-Sephadex C-25, ECTEOLA-cellulose (not form B), Con A-Sepharose 4B and Sephadex G-200. The purification data at the end of this extensive procedure are summarised in Table II. Yields were not important as the emphasis was on the relative behaviour of the two hexosaminidase activities. A comparison of the data indicates lower enzyme yields and degrees of purification for β-*N*-acetylgalactosaminidase than for β-*N*-acetylglucosaminidase. The ratio of the specific acitivity of unpurified β-*N*-acetylglucosaminidase to unpurified β-*N*-acetylgalactosaminidase was nearly unity whereas the ratios for the purified forms A, B and I were 5 or greater.

*PH vs. activity profiles*

The pH optima of the different forms of the two hexosaminidases were determined using citrate/phosphate buffers from pH 2.5 to 8.0 and glycine buffers from pH 8.0 to 10.0 [22]. The pH optima are in agreement with the value of 4.2 found in the standard assay buffer (citrate).

*$K_m$ and V values*

The $K_m$ and $V$ values for both the β-*N*-acetylhexosaminides that were used as substrates for the different forms of the enzymes are given in Table III. The values are lower for the galactosaminide substrate, and for each activity, the $K_m$ values for the three forms differ.

*Kinetics of the inhibition by substrate analogues*

Analysis of the rate data at different inhibitor concentrations showed that both types of inhibitor, the *N*-acetylhexosamines and the *N*-acetylhexosaminolactones, acted competitively with respect to both substrates. The results are given as $K_i$ values in Table IV. The *N*-acetylhexosaminolactones were in every

TABLE I

PRELIMINARY PURIFICATION OF THE β-*N*-ACETYLGLUCOSAMINIDASE ACTIVITY FROM RAT COLONIC MUCOSA

| Enzyme | Total protein (mg) | Total activity (μmol/h) | Specific activity (nmol/h per mg protein) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Supernatant after homogenisation | 9540 | 40.5 | 4.2 | 1 | 100 |
| Sepharose 6B | 451 | 34.4 | 76 | 18 | 85 |
| Sephadex G-200 | 118 | 27.1 | 230 | 54 | 67 |
| First DE-52 | | | | | |
| A | 22 | 12.1 | 550 | 130 | 30 |
| B | 13 | 8.3 | 640 | 150 | 21 |
| I | 8 | 3.8 | 470 | 110 | 10 |

TABLE II

SUMMARY OF THE PURIFICATION DATA FOR THE THREE FORMS OF BOTH β-*N*-ACETYLHEXOSAMINIDASE ACTIVITIES

The data for forms A, B and I are after the final Sephadex G-200 step (see text).

| Enzyme | Total protein (mg) | Total activity (μmol/h) | Specific activity (nmol/h per mg protein) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| β-*N*-Acetylglucosaminidase | | | | | |
| Supernatant | 9500 | 40.5 | 4 | 1 | 100 |
| A | 0.20 | 4.21 | 21 500 | 5060 | 10.4 |
| B | 0.17 | 3.72 | 22 300 | 5260 | 9.2 |
| I | 0.06 | 1.13 | 18 800 | 4440 | 2.8 |
| β-*N*-Acetylgalactosaminidase | | | | | |
| Supernatant | 9500 | 34.6 | 4 | 1 | 100 |
| A | 0.20 | 0.83 | 4 210 | 1160 | 2.4 |
| B | 0.17 | 0.74 | 4 420 | 1220 | 2.1 |
| I | 0.06 | 0.23 | 3 760 | 1040 | 0.7 |

case more effective inhibitors than the *N*-acetylhexosamines and within these two groups, the galactosyl derivatives were more effective than the corresponding glucosyl derivatives, as judged by the fact that the $K_i$ values were significantly lower ($P < 0.001$). However, when the data for the two different substrates were compared, it was seen that the $K_i$ value for each of these inhibitors with respect to *p*-nitrophenyl-β-*N*-acetylglucosaminide was significantly lower ($P < 0.001$—$0.005$) than the value with respect to *p*-nitrophenyl-β-*N*-acetylgalactosaminide.

*Effect of divalent cations and various anions*

The activities of the various forms of both hexosaminidases remained unchanged when enzyme samples were treated with 1.0—5.0 mM chloride salts

TABLE III

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES FOR THE DIFFERENT FORMS OF THE β-*N*-ACETYLHEXOSAMINIDASES

Values are given as means ± S.E. of 20 experiments. Units for $K_m$ are mM; bracketed values are *V* as nmol of product/h per mg protein.

| Enzyme form | Substrate | |
|---|---|---|
| | *p*-Nitrophenyl-β-*N*-acetylglucosaminide | *p*-nitrophenyl-β-*N*-acetylgalactosaminide |
| A | 0.770 ± 0.146 (0.666 ± 0.107) | 0.345 ± 0.060 (0.081 ± 0.018) |
| B | 0.355 ± 0.084 (0.564 ± 0.165) | 0.148 ± 0.068 (0.052 ± 0.010) |
| I | 0.550 ± 0.100 (0.429 ± 0.176) | 0.216 ± 0.035 (0.044 ± 0.017) |

The $K_m$ and *V* values for the galactosaminide substrate are significantly lower ($P < 0.001$) than the corresponding values for the glucosaminide substrate.

TABLE IV

$K_i$ VALUES OF SUBSTRATE ANALOGUES AS INHIBITORS OF β-*N*-ACETYLHEXOSAMINIDASE ACTIVITY

Values are given as means ± S.E. of 10 experiments and represent the inhibition constant affecting the slope of a double reciprocal plot [10].

| Enzyme form | Inhibitor | | | |
|---|---|---|---|---|
| | 1 *N*-Acetyl glucosamine (mM) | 2 *N*-Acetyl-galactosamine (mM) | 3 *N*-Acetyl-glucosaminolactone (μM) | 4 *N*-Acetyl-galactosaminolactone (μM) |
| Substrate: *p*-nitrophenyl-β-*N*-acetylglucosaminide | | | | |
| A | 4.34 ± 1.01 | 0.491 ± 0.065 | 2.84 ± 0.62 | 1.81 ± 0.37 |
| B | 3.61 ± 0.85 | 0.405 ± 0.042 | 2.07 ± 0.53 | 1.06 ± 0.41 |
| I | 4.02 ± 1.15 | 0.442 ± 0.035 | 2.62 ± 0.75 | 1.41 ± 0.29 |
| Substrate: *p*-nitrophenyl-β-*N*-acetylgalactosaminide | | | | |
| A | 9.40 ± 2.23 | 2.90 ± 0.45 | 8.48 ± 2.05 | 4.96 ± 1.07 |
| B | 3.93 ± 0.77 | 1.86 ± 0.25 | 3.68 ± 0.48 | 1.89 ± 0.42 |
| I | 6.73 ± 1.32 | 2.63 ± 0.38 | 4.66 ± 1.01 | 2.49 ± 0.28 |

The values in column 2 are significantly lower ($P < 0.001$) than the corresponding values in column 1. The values in column 4 are significantly lower ($P < 0.001$) than the corresponding values in column 3. Note the different units for the lactone $K_i$ values.

of $Ba^{2+}$, $Ca^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Mn^{2+}$ or $Pb^{2+}$ or with 10—100 mM sulphate, succinate or maleate. Phosphate up to 0.4 M did not affect the enzyme activities. In these experiments, the enzyme samples which were normally stored in phosphate buffer were dialysed overnight against 0.1 M 2-(*N*-morpholino)-ethanesulphonate buffer (pH 6.0) before use. Arsenite, iodoacetate and *N*-ethylmaleimide up to 5.0 mM also failed to affect the enzyme activities.

Acetate was found to be a competitive inhibitor of both enzyme activities. The $K_i$ values of acetate with respect to the galactosaminide substrate were significantly higher ($P < 0.001$) than those for the glucosaminide substrate for all three enzyme forms (Table V). The presence of acetate did not change the pH optimum of β-*N*-acetylgalactosaminidase in contrast to the report of a change from pH 4.2 to 6.0 by Frohwein and Gatt [8] for the calf brain enzyme.

*Effect of heavy metal compounds*

$HgCl_2$, $AgNO_3$ and *p*-hydroxymercuribenzoate were effective inhibitors of the two activities. $K_i$ values are reported in Table V. The kinetics of inhibition by $AgNO_3$ and $HgCl_2$ were statistically more consistent with their being competitive inhibitors with respect to both substrates than non-competitive. For any one enzyme form, the $K_i$ values of $AgNO_3$ for *p*-nitrophenyl-β-*N*-acetylglucosaminide were significantly different ($P < 0.005$) from those for *p*-nitrophenyl-β-*N*-acetylgalactosaminide, higher in the case of form A but lower for the other two enzyme forms.

*p*-Hydroxymercuribenzoate showed some variation in the type of inhibition. At pH 4.2 it inhibited the three forms of β-*N*-acetylglucosaminidase competitively, form A having a significantly higher ($P < 0.001$) $K_i$ value than forms B

TABLE V

$K_i$ VALUES OF INHIBITORS OF β-*N*-ACETYLHEXOSAMINIDASE ACTIVITY

Values are given as means ± S.E. of three experiments.

| Inhibitor | pH * | Enzyme form | | |
|---|---|---|---|---|
| | | A | B | I |
| Substrate: *p*-nitrophenyl-β-*N*-acetylglucosaminide | | | | |
| Acetate (mM) | 4.2 | 10.81 ± 1.09 | 11.77 ± 1.59 | 8.74 ± 1.17 |
| $HgCl_2$ (μM) | 4.2 | 0.005 ± 0.003 | 0.0006 ± 0.0002 | 0.0007 ± 0.0003 |
| $AgNO_3$ (μM) | 4.2 | 0.133 ± 0.048 | 0.003 ± 0.001 | 0.004 ± 0.001 |
| *p*-Hydroxy-mercuri-benzoate (μM) | 4.2 | 0.009 ± 0.001 | 0.003 ± 0.002 | 0.002 ± 0.001 |
| | 5.6 | 0.001 ± 0.0004 ($K_{is}$) ** 0.003 ± 0.001 ($K_{ii}$) ** | (no inhibition up to 12.5 M) | 0.003 ± 0.0004 |
| | 7.0 | 0.001 ± 0.0004 ($K_{is}$) 0.003 ± 0.001 ($K_{ii}$) | 0.185 ± 0.041 | 0.003 ± 0.0005 |
| Substrate: *p*-nitrophenyl-β-*N*-acetylgalactosaminide | | | | |
| Acetate (mM) | 4.2 | 28.7 ± 7.2 | 22.7 ± 4.6 | 48.8 ± 5.4 |
| $HgCl_2$ (μM) | 4.2 | 0.003 ± 0.001 | 0.0014 ± 0.0007 | 0.0008 ± 0.0004 |
| $AgNO_3$ (μM) | 4.2 | 0.023 ± 0.008 | 0.006 ± 0.003 | 0.020 ± 0.003 |
| *p*-Hydroxy-mercuri-benzoate (μM) | 4.2 | 0.004 ± 0.0004 | 0.021 ± 0.014 | 0.004 ± 0.001 |
| | 5.6 | 0.003 ± 0.0005 | 0.024 ± 0.017 | 0.002 ± 0.0004 |
| | 7.0 | 0.003 ± 0.0005 | 0.035 ± 0.013 | 0.001 ± 0.0003 |

* pH at which incubation (at 37°C for 15 min) with inhibitor was carried out before assaying at pH 4.2.

** $K_{is}$ and $K_{ii}$ are inhibition constants affecting, respectively, the slope and intercept of double reciprocal plots [10].

and I. At pH 5.6, 12.5 μM *p*-hydroxymercuribenzoate failed to inhibit form B, and the inhibition of form A was statistically more consistent with non-competitive than competitive inhibition, although the reverse held for form I. At pH 7.0, the B and I forms were inhibited competitively, while inhibition of form A was non-competitive. On the other hand, the types of inhibition for the β-*N*-acetylgalactosaminidase forms were all competitive, the $K_i$ values for the B form being significantly greater ($P < 0.001$) at all three pH values than those for the A and I forms.

*Kinetics of mixed substrates reactions*

The different forms of the β-*N*-acetylhexosaminidases from rat colonic mucosa were used in these experiments in which the rate of appearance of the common product, *p*-nitrophenol, was measured with *p*-nitrophenyl-β-*N*-acetylglucosaminide and *p*-nitrophenyl-β-*N*-acetylgalactosaminide as joint substrates in varying proportions. The $K_m$ and $V$ values for each substrate alone and the $V$ values for the mixture were calculated from the initial velocity measurements (Table VI). The possibility of competition by the two substrates for the same active sites on the enzyme molecules was tested using Eqn. 1 [23].

$$\frac{K_a}{\alpha K_b} = \frac{V_a - V_m}{V_m - V_b} \quad (1)$$

TABLE VI

KINETIC PARAMETERS DETERMINED FROM TOTAL VELOCITY MEASUREMENTS FOR THE DIFFERENT ENZYME FORMS WITH AND WITHOUT BOTH SUBSTRATES PRESENT SIMULTANEOUSLY

Values given are the average of three experiments. Units are: $K_a$, $K_b$, mM; $V_a$, $V_b$, $V_m$, nmol of product/h per mg protein. $K_a$, $K_b$, $V_a$, $V_b$, $\alpha$ and $V_m$ are defined in Eqn. 1.

| Enzyme form | $K_a$ | $V_a$ | $K_b$ | $V_b$ | $\alpha$ | $V_m$ | |
|---|---|---|---|---|---|---|---|
| | | | | | | calculated according to Eqn. 1 | observed |
| A | 0.738 | 0.389 | 0.247 | 0.088 | 1.0 | 0.163 | 0.086 |
| | | | | | 5.0 | 0.277 | 0.157 |
| | | | | | 0.2 | 0.107 | 0.055 |
| B | 0.249 | 0.266 | 0.080 | 0.032 | 1.0 | 0.089 | 0.035 |
| | | | | | 5.0 | 0.176 | 0.089 |
| | | | | | 0.2 | 0.046 | 0.017 |
| I | 0.550 | 0.149 | 0.175 | 0.052 | 1.0 | 0.075 | 0.022 |
| | | | | | 5.0 | 0.112 | 0.044 |
| | | | | | 0.2 | 0.058 | 0.022 |

where $K_a$, $V_a$, $K_b$ and $V_b$ are the $K_m$ and $V$ values of *p*-nitrophenyl-β-*N*-acetylglucosaminide (a) and *p*-nitrophenyl-β-*N*-acetylgalactosaminide (b) and $V_m$ is the maximum velocity observed when substrates a and b are mixed in the ratio $\alpha$ = a/b. Values of $V_m$ calculated from this equation were always higher than those observed (Table VI). This consistent discrepancy rules out pure competition of the substrates for a common active site.

More information becomes available if the transformation of each substrate can be followed separately, and to this end, phenyl and *p*-nitrophenyl derivatives were employed. The reaction with phenyl-β-*N*-acetylglucosaminide (substrate a) was followed by measuring phenol production, and the reaction with *p*-nitrophenyl-β-*N*-acetylgalactosaminide (substrate *b*) by *p*-nitrophenol production. In each reaction, the addition of the other compound gave kinetics that

TABLE VII

MICHAELIS AND INHIBITION CONSTANTS FOR THE DIFFERENT ENZYME FORMS DETERMINED BY MEASURING RATES DUE TO ONE SUBSTRATE IN THE PRESENCE OF BOTH

Substrates are a, phenyl-β-*N*-acetylglucosaminide and b, *P*-nitrophenyl-β-*N*-acetylgalactosaminide. Values are given in mM units and are the average of three experiments. $K_a$, $K_{ia}$, $K_b$ and $K_{ib}$ are defined in Eqn. 2. Inhibition was competitive.

| Enzyme form | Substrate | | | | | |
|---|---|---|---|---|---|---|
| | a, phenyl-β-*N*-acetylglucosaminide | | | b, *p*-nitrophenyl-β-*N*-acetylgalactosaminide | | |
| | $K_a$ | $K_{ia}$ | $K_a/K_{ia}$ | $K_b$ | $K_{ib}$ | $K_{ib}/K_b$ |
| A | 0.949 | 3.084 | 0.308 | 0.191 | 0.042 | 0.220 |
| B | 0.955 | 1.293 | 0.739 | 0.088 | 0.085 | 0.966 |
| I | 0.974 | 1.850 | 0.526 | 0.079 | 0.025 | 0.316 |

were consistent with competitive inhibition, and could be analysed to give a value for the true $K_m$ and a value for the inhibition constant. The true $K_m$ had the same value in the presence or absence of inhibitor.

Table VII shows that for a particular substrate, its $K_m$ was different from its $K_i$, thus confirming the above conclusion that the two substrates are not simply competing for common active sites.

Walker et al. [6] have shown that if the displacement of one substrate by the other from its complex with the enzyme is included in the common active site mechanism, the following relationship holds, even though the individual Michaelis constants differ from the inhibition constants:

$$\frac{K_a}{K_{ia}} = \frac{K_{ib}}{K_b} \tag{2}$$

where $K_a$ and $K_b$ are the Michaelis constants for the two substrates, $K_{ia}$ is the inhibition constant for substrate when it competitively inhibits the reaction of b as substrate, and $K_{ib}$ is the inhibition constant for substrate b in the reaction of a as substrate. In Table VII these ratios are calculated but are seen to be different for each of the enzyme forms.

*Inactivation of β-N-acetylglucosaminidase by reagents reported able to convert forms A and I into B-like forms*

*(1) Neuraminidase.* All three forms of the enzyme when incubated with 10—100 units of neuraminidase for 18 h at 37° C showed about 10—15% inactivation similar to the control samples. Differences in the microbial origin of neuraminidase and in the commercial source caused no difference in effect on the enzyme forms.

*(2) p-Hydroxymercuribenzoate.* The inactivation of the enzyme by *p*-hydroxymercuribenzoate was dependent on pH, and the addition of cysteine up to 10 mM before or after treatment did not prevent or reverse the effect. At pH 4.2, 5.6 and 7.0, treatment with 5 μM *p*-hydroxymercuribenzoate for 15 min at 37° C was sufficient to reduce the activity of forms A and I to 50% or less. A higher concentration was needed for form B and the relationship between activity remaining and *p*-hydroxymercuribenzoate concentration was sigmoid rather than hyperbolic at all three pH values.

The enzyme showed a biphasic decay at 37° C (Fig. 1), the first stage being very rapid at all three pH values for forms A and I while form B was relatively stable, especially at pH 5.6 where prolonged incubation of up to 18 h was necessary before the activity was reduced to 10%, an effect accomplished within 15 min for forms A and I.

*(3) Thimerosal.* The effect of thimerosal appeared to be independent of pH over the range 4.2—7.0 where no difference in the degree and profile of the enzyme inactivation was found. Neither extensive dialysis of the treated samples nor incubation with glutathione at 10 times the thimerosal concentration reversed the inactivation. However, addition of glutathione at these concentrations prior to the thimerosal treatment prevented inactivation.

*(4) $HgCl_2$ and $AgNO_3$.* $HgCl_2$ was a more rapid inactivator of the enzyme than $AgNO_3$ and inactivation of all three forms by these reagents was a simple first-order decay process and independent of pH. Addition of cysteine up to

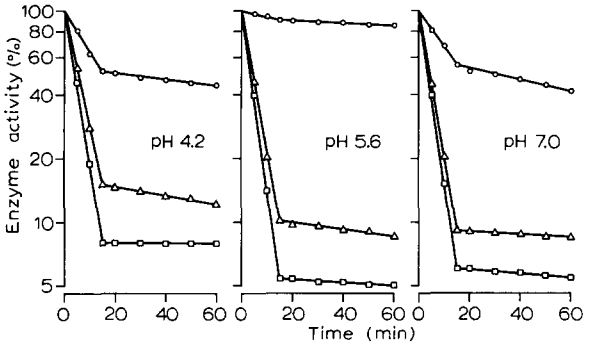


Fig. 1. The time course of the inactivation by *p*-hydroxymercuribenzoate of the β-*N*-acetylglucosaminidases. Samples of form A (△———△), B (○———○) and I (□———□) were incubated at 37°C in the presence of 10 μM *p*-hydroxymercuribenzoate at pH 4.2, 5.6 and 7.0. Aliquots were than taken at different time intervals and the pH adjusted where necessary to 4.2 for assay of activity. Activities were expressed as a percentage of the activity of a control sample kept at pH 4.2 without *p*-hydroxymercuribanzoate.

10 mM before or after treatment with $HgCl_2$ did not prevent or reverse the inactivation. The rate of inactivation of the three forms decreased in the order A, I, B in the case of $HgCl_2$, and I, B, A in the case of $AgNO_3$.

*Effect of p-nitrophenyl-β-N-acetylhexosaminides on the inactivation of β-N-acetylglucosaminidase by neuraminidase and SH reagents*

Pretreatment of the enzyme with *p*-nitrophenyl-β-*N*-acetylglucosaminide and its presence in the inactivation mixture did not affect the inactivation of β-*N*-acetylglucosaminidase activity by neuraminidase, $HgCl_2$, $AgNO_3$ or thimerosal. However, in the case of *p*-hydroxymercuribenzoate inactivation, addition of this substrate at a concentration 50 times greater than *p*-hydroxymercuribenzoate reduced the inactivation by 15%. In similar experiments, the presence of the galactosaminide substrate by contrast increased the *p*-hydroxymercuribenzoate inactivation of the glucosaminidase activity (measured with phenyl-β-*N*-acetylglucosaminide as substrate). No difference in the behaviour of the three forms of the enzyme was observed in these experiments.

*Inactivation of β-N-acetylgalactosaminidase activity*

The extent and pattern of the inactivation of the β-N-acetylgalactosaminidase activity by neuraminidase, thimerosal, $HgCl_2$ and $AgNO_3$ were similar to the results described above for the β-*N*-acetylglucosaminidase activity. Some differences were however observed in the response to *p*-hydroxymercuribenzoate. Concentrations of *p*-hydroxymercuribenzoate about 20 times higher were required to produce inactivation of the galactosaminidase activity similar in degree to that of the glucosaminidase activity. The *p*-hydroxymercuribenzoate inactivation of β-*N*-acetylgalactosaminidase form B showed a simple first-order decay whereas the effect with forms A and I was biphasic (Fig. 2). Form B of the enzyme was considerably more resistant to the action of *p*-hydroxymercuribenzoate than forms A and I. Pretreatment with *p*-nitrophenyl-β-*N*-acetylgalactosaminide and its presence during the inactivation reaction at a

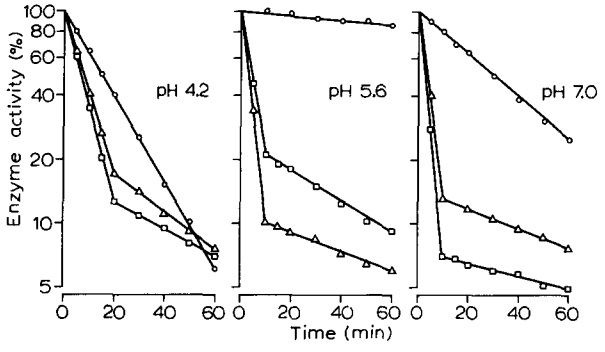


Fig. 2. The time course of the inactivation by *p*-hydroxymercuribenzoate of the β-*N*-acetylgalactosaminidases. Samples of form A (△———△), B (○———○) and I (□———□) were incubated at 37°C in the presence of 200 μM *p*-hydroxymercuribenzoate at pH 4.2, 5.6 and 7.0. Other details are as in Fig. 1.

concentration 10 times that of the *p*-hydroxymercuribenzoate reduced the inactivation by about 25%. The addition of phenyl-β-*N*-acetylglucosaminide, a substrate for the other enzyme activity, did not however alter the inactivation of the galactosaminidase activity.

*Inconvertibility of forms A and I into B-like forms*

The reagents used in attempts to convert forms A and I into forms resembling B included neuraminidase, *p*-hydroxymercuribenzoate, thimerosal, $HgCl_2$ and $AgNO_3$. The concentrations of these reagents and other experimental conditions are given in Table VIII. In addition, forms A and I were heated at 50°C for 15 min prior to analysis. The identity of the reaction products and the conversion of forms A and I into B-like forms was examined by DEAE-cellulose ion-exchange chromatography, kinetics of thermal inactivation at 50°C, and in some cases by other kinetic properties of the enzyme.

*(1) DEAE-cellulose chromatography of the treated A and I forms.* DE-52 chromatographic profiles of the enzyme activity for forms A and I after treatment with neuraminidase, thimerosal, *p*-hydroxymercuribenzoate, $HgCl_2$ and $AgNO_3$ and after heating at 50°C for 15 min were identical to those of the corresponding untreated forms as regards enzyme activity, and in particular no

TABLE VIII

EXPERIMENTAL CONDITIONS USED IN ATTEMPTS TO CONVERT HEXOSAMINIDASES A AND I INTO FORMS RESEMBLING B

Buffer systems: A, 50 mM phosphate/citrate containing 20 mM $CaCl_2$, pH 5.6; B, 50 mM phosphate/citrate, pH 4.2; C, 50 mM phosphate/citrate, pH 5.6; D, 50 mM phosphate/citrate, pH 7.0.

| Reagent | Concentration | Buffer system | Time of incubation (h) |
|---|---|---|---|
| Neuraminidase | 100 units | A | 18 |
| *p*-Hydroxymercuribenzoate | 5 μM | B,C,D | 1,18 |
| Thimerosal | 1 mM | B,C,D | 1,18 |
| $HgCl_2$ | 1.5 μM | B,D | 1 |
| $AgNO_3$ | 10 μM | B,D | 1 |

activity was eluted from any column at zero NaCl concentration. Scanning of the column fractions for protein showed a single peak coinciding with enzyme activity in all cases. An increased NaCl gradient up to 0.45 M did not cause elution of any further enzyme activity or protein. The recovery of enzyme activity was always between 90 and 95% of that initially applied to the column.

*(2) Thermal inactivation at 50°C of the treated A and I forms.* The kinetics of thermal inactivation of the enzyme samples were examined immediately after treatment with the above reagents and after the subsequent DEAE-cellulose chromatography. The thermal inactivation profiles before and after chromatography were identical, and similar to those of the untreated samples but bore no resemblance to those expected for form B.

*(3) Kinetic properties of the treated A and I forms.* Enzyme kinetics experiments were performed on samples treated with neuraminidase or thimerosal and subsequently passed through DE-52. A comparison of the $K_m$ values of both *p*-nitrophenyl substrates and the $K_i$ values of the four substrate analogue inhibitors with the data in Tables III and IV indicated no significant difference that would suggest conversion to a B-like form.

## Discussion

The existence of a form of the β-*N*-acetylhexosaminidases from rat colonic mucosa with mobility properties intermediate between those of the A and B forms [3] has been confirmed by DE-52 chromatography. Similar intermediate forms have been reported in some human tissues and other biological fluids [24—27]. Purification of each of the three forms by a number of different chromatographic methods, and concomitant activity measurements with either the glucosaminide or galactosaminide substrate, show that the two activities are inseparable, in agreement with the observations of several other laboratories [4,5,9,14]. The percentage yields and degrees of purification of the three forms of β-*N*-acetylglucosaminidase were similar to these other reports and always higher than those of β-*N*-acetylgalactosaminidase. The specific activity ratio in the supernatant after homogenisation had a value less than 2, in agreement with our previously reported values [20,28] but in contrast to those for other tissues [4,29,30]. The ratio rose to about 5 for the material from the final Sephadex G-200 column (Table II), a value in agreement with that of Verpoorte [4] but not other workers [29,30].

Hexosaminidases from other tissues have been shown to be glycoproteins. The data on the enzymes from rat colonic mucosa suggest that β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities either belong to two different glycoprotein molecules which however have identical chromatographic and electrophoretic behaviour or to a glycoprotein molecule composed of subunits which have evolved two kinds of active centre, each specific for the hydrolysis of one kind of hexosaminide substrate. The second postulate would require changes in the interaction between the subunits during purification so that the two kinds of active site were affected differently, giving rise to the change in specific activity ratio that is observed upon purification. The one constant factor among the two activities and the different isoenzymes was the optimum pH.

The kinetic results reported here support the model of a single molecule with two kinds of active site. In agreement with previous proposals of a common active site (for example, refs. 4, 6 and 9), we found that the $K_m$ and the $V$ values of *p*-nitrophenyl-β-*N*-acetylgalactosaminide were significantly lower than the values obtained for *p*-nitrophenyl-β-*N*-acetylglucosaminide. However, the fact that *N*-acetylgalactosamine and *N*-acetylgalactosaminolactone were found to be more effective competitive inhibitors than the glucose analogues for both substrates, and that the $K_i$ values for all these inhibitors for *p*-nitrophenyl-β-*N*-acetylgalactosaminide were significantly higher than the corresponding values for *p*-nitrophenyl-β-*N*-acetylglucosaminide cannot be explained on the basis of pure competition at a common active site. A common site would require equal $K_i$ values for a particular inhibitor regardless of the nature of the substrate. Similar results have been reported for the rat kidney β-*N*-acetylhexosaminidase [6].

The kinetics data on the inhibition of the two enzymic activities by $AgNO_3$ and *p*-hydroxymercuribenzoate showed some interesting features. There was a significant variation in the $K_i$ values for $AgNO_3$, a competitive inhibitor for both acetylhexosaminidases. *p*-Hydroxymercuribenzoate also showed considerable differences in the $K_i$ values for both activities and in the mode of inhibition. Such differences in the inhibition kinetics of the two activities were not compatible with the assumption of a single active site for the two substrates.

The inhibitory effect of $AgNO_3$, *p*-hydroxymercuribenzoate and thimerosal on human β-*N*-acetylglucosaminidase A has been reported [16]. These thiol reagents were also found to convert form A into a form resembling form B [16,17]. Such effects of thiol reagents on the *N*-acetylhexosaminidases suggest the presence of sulphydryl grops, though not necessarily in the active site.

Analysis of the reaction rates with both *p*-nitrophenyl substrates present did not yield results compatible with the mechanism described by Dixon and Webb [23] for a single type of active site catalysing two reactions simultaneously. By arranging to follow the reactions of the two substrate types independently, it was possible to confirm that such a mechanism does not hold, nor the slightly more complex one described by Walker et al. [6] that involves an additional displacement reaction.

Walker et al. [6] have also described a mechanism in which there are two different types of active site, each specific for the transformation of one substrate, but able to be inhibited competitively by the other substrate [6]. This model predicts the same experimental findings as for their mechanism of a commmon active site plus the extra displacement reaction, except that the relationship of Eqn. 2 no longer holds. The present results shown in Table VII do not fit Eqn. 2 and are therefore compatible with such a model, which is the same as the one we have suggested on the basis of the purification results. Thus the same protein molecule carries both enzyme activities, but at different active sites, each of which can be inhibited by the substrate for the other kind of site. The values of the inhibition constants in Table VII show that the glucosaminide compound is a poorer inhibitor of the galactosaminidase activity than the galactosaminide is of the glucosaminidase.

The A, B and I forms of the rat colon hexosaminidases differ in their kinetic parameters and inhibition constants when the results for a particular substrate

are considered (Tables III—V). Their behaviour upon inactivation by SH reagents, whether monitored as glucosaminidase or galactosaminidase activity, adds to the evidence of differences among them. Such differences may reflect differences in the modes of action of the reagents as well as differences in the SH groups of the enzyme forms. The enzyme inhibition by thimerosal, $HgCl_2$ and $AgNO_3$ may be due to a conformational changed adversely affecting the catalytic sites but induced by the new and perhaps distant sulphur substituents. The present data confirm similar observations made on beef spleen [4] and human placental enzymes [16]. Our observation that substrates partially protect against the *p*-hydroxymercuribenzoate inactivation of the rat colonic enzymes may be interpreted to mean that the SH group reacting with *p*-hydroxymercuribenzoate is at or near the active centres. An alternative explanation is that the position and hence the accessibility of such SH groups is altered as a consequence of the conformational change in the enzyme due to substrate binding. This second explanation is consistent with the absence of protection by substrates against inactivation by the smaller $AgNO_3$ and $HgCl_2$. These compounds are however apparently competitive inhibitors of the kinetics, and this must be due to their removal of a fraction of active enzyme that can then no longer bind substrate.

Another aspect of the inactivation by *p*-hydroxymercuribenzoate supports the hypothesis of different active sites. The glucosaminidase activity is protected by a glucosaminide substrate but rendered more sensitive to inactivation by the corresponding galactose derivative; the galactosaminidase activity is protected by the galactosaminide substrate, but not affected by the corresponding glucose derivative. A common active site would of course result in both activities being affected identically by any one protecting agent. A comparison of Figs. 1 and 2 shows that the patterns for the inactivation kinetics are different for the two activities as well.

The data in the present paper clearly indicate that the rat colonic form A cannot be converted into a B-like form by the action of neuraminidase or sulphydryl group reagents, in contrast to previous reports on these enzymes from other rat tissues and human placenta [1,11—18]. Apart from the fact that the rat colonic enzymes A and I are strongly inactivated by these reagents, they maintain their original chromatographic, thermostability and kinetics properties after the treatment. This failure to alter the properties suggests some structural differences between the rat colonic enzymes on the one hand and those enzymes that have been shown to be convertible by neuraminidase or SH reagents on the other. The inactivation itself may be explained on the assumption that the subunits of rat colonic hexosaminidase A undergo some rearrangement in response to SH reagents. As the subunit interactions involve specific residues at the contact interfaces, the thiol-linked conformational changes could displace these residues, and this could affect the formation of the native ensemble and result in the inactivation of the enzyme.

Further evidence for structural differences in the rat colonic enzymes compared with those from other sources lies in the finding that cysteine does not reverse the *p*-hydroxymercuribenzoate inactivation in contrast to the situation with the beef spleen enzyme [4]. Heating at 50°C failed to convert from A into form B, unlike the transformation reported by Tallman and coworkers [5] for

the human placental enzyme and ascribed to a conformation change.

It thus appears that the structural relationships among the rat colonic enzymes are different from those proposed for the enzymes from other sources and in particular the difference between forms A and B cannot be described in terms of the subunit reassociation model, mediated by SH changes, that has been proposed for the human kidney and placental enzymes [17,18], nor in terms of different conformational states [5]. In order to fully understand the interrelationship among the forms of this enzyme, further investigation of the structure and interaction of the subunits will be necessary.

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