Studies on Glucosaminidase

5. KIDNEY *N*-ACETYL- β -GLUCOSAMINIDASE AND *N*-ACETYL- β -GALACTOSAMINIDASE*

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The enzyme *N*-acetyl- β -D-galactosaminidase has been studied in a rat-kidney preparation which was known to have *N*-acetyl- β -D-glucosaminidase activity (Pugh, Leaback & Walker, 1957). It was not found possible to separate the two activities by various fractionation procedures. The two *p*-nitrophenyl hexosaminides which were used as substrates competed but not in the simple manner found with a testicular extract (Woollen, Heyworth & Walker, 1961).

A preliminary account of part of this work has appeared (Woollen, Heyworth & Walker, 1959).

MATERIALS AND METHODS

Enzyme preparation. Fresh rat kidney was weighed and ground for 1 min. with water in a glass homogenizer and brought to pH 4.5 with 0.05M-sodium citrate-citric acid buffer and a final concentration of 10% (w/v). The mixture was centrifuged (1500g) and the supernatant allowed to stand for 1 day at 4° before use. Dilutions were made from this stock solution in 0.05M-citrate buffer, pH 4.5, when required, immediately before use.

Attempts to use enzyme preparations which had been partially purified by ammonium sulphate fractionation were made difficult by the occasional inconsistency of duplicate determinations of enzymic activities.

Substrates, inhibitors and assay procedure. These are described by Woollen *et al.* (1961). Except where otherwise indicated 0.1 ml. of enzyme solution was incubated with substrate in 0.05M-citrate buffer, pH 4.52, for 30 min. at 37° in a total volume of 1.0 ml. These conditions were chosen after initial experiments described under Results.

In experiments where mixtures of phenyl and *p*-nitrophenyl substrates were used it was possible to estimate the liberated phenol and *p*-nitrophenol separately since *p*-nitrophenol gives no colour under the conditions of phenol estimation (Folin & Denis, 1912) and phenol gives no colour under the conditions of *p*-nitrophenol estimation.

RESULTS

Effect of pH, enzyme concentration and time of incubation

Fig. 1 shows the effect of pH on *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities.

* Part 4: Leaback & Walker (1961).

The general shape of the pH curves was similar for the two activities.

Both enzyme activities were proportional to the enzyme present over a fourfold range of enzyme concentration with the substrates *p*-nitrophenyl *N*-acetylglucosaminide (3.6 mM) and *p*-nitrophenyl *N*-acetylgalactosaminide (2.2 mM). With the same substrates both activities were proportional to time of incubation over the period tested (1 hr.).

Attempts to separate the two activities

Fractionation and partial inactivation of the crude kidney-enzyme preparation were carried out in the same way as with a testicular-enzyme preparation (Woollen *et al.* 1961). The ratios of *N*-

Fig. 1. Effect of pH on enzyme activities in the presence of 3.6 mM-*p*-nitrophenyl *N*-acetylglucosaminide and 0.25% (w/v) of enzyme (O) or 2.2 mM-*p*-nitrophenyl *N*-acetylgalactosaminide and 1.25% (w/v) of enzyme (●). For other conditions see text.

Table 1. *Effect of ammonium sulphate fractionation on N-acetylglucosaminidase and N-acetylgalactosaminidase activities*

Activities were measured with either 2.2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate. For other conditions see text.

Enzyme preparation	<i>N</i> -Acetylglucosaminidase (% of activity of untreated preparation)	Ratio <i>N</i> -acetylglucosaminidase: <i>N</i> -acetylgalactosaminidase activities
Untreated (NH ₄) ₂ SO ₄ fractions:†	100.0*	8.2
0-30 %	6.7	8.8
30-40 %	3.3	8.0
40-50 %	19.3	8.3
50-60 %	8.0	7.7

* 66.6 µg. of *p*-nitrophenol liberated/tube.

† Precipitated between the indicated limits of percentage saturation with (NH₄)₂SO₄.

Table 2. *Adsorption of N-acetylglucosaminidase and N-acetylgalactosaminidase activities by calcium phosphate gel*

Residual activities in the supernatants were measured with either 2.2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate. For other conditions see text.

Total gel added (ml./10 ml. of enzyme solution)	<i>N</i> -Acetylglucosaminidase (% of initial activity)	Ratio <i>N</i> -acetylglucosaminidase: <i>N</i> -acetylgalactosaminidase activities
0	100*	8.5
0.5	91	8.8
0.6	44	9.0
0.7	34	9.3
0.8	17	8.7
0.9	6	9.0

* 32.9 µg. of *p*-nitrophenol liberated/tube.

Table 3. *Effect of partial heat inactivation on N-acetylglucosaminidase and N-acetylgalactosaminidase activities*

The enzyme preparation was preincubated in 0.05 M-citrate buffer, pH 4.5, at 56° for various times in the absence of substrate. Activities were then measured with 2.2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide in the usual way.

Period of preincubation (min.)	<i>N</i> -Acetylglucosaminidase (% of initial activity)	Ratio <i>N</i> -acetylglucosaminidase: <i>N</i> -acetylgalactosaminidase activities
0	100*	7.9
15	52	8.1
30	29	7.0
60	15	6.9

* 78.6 µg. of *p*-nitrophenol liberated/tube.

acetylglucosaminidase: *N*-acetylgalactosaminidase, measured under standard conditions, were constant both on ammonium sulphate fractionation (Table 1) and with progressive adsorption (Table 2) on calcium phosphate gel (Keilin & Hartree, 1951). On partial heat inactivation to 15 % of the initial activity the ratio again remained constant (Table 3).

Kinetic experiments

The above results indicated that a single enzyme protein in rat kidney had both *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities. Kinetic experiments were then undertaken to study the nature of the combination of the enzyme with the respective substrates.

Effect of substrate concentration. K_m and V_{max} values (Table 4) were determined according to Lineweaver & Burk (1934) from typical straight-line plots for each of the four substrates.

Competitive behaviour. With the *p*-nitrophenyl substrates, both activities were competitively inhibited by *N*-acetylglucosamine, 2-acetamido-2-deoxygluconolactone and phenyl *N*-acetyl-β-D-glucosaminide and by the corresponding galactosamine derivatives (cf. Woollen *et al.* 1961). The K_i values (Table 5), however, show marked differences when measured against the two respective substrates. By comparing Tables 4 and 5 it can be seen that the K_i values for the two phenyl *N*-acetylhexosaminides agree more closely with the K_m values when K_i is measured in the presence of the analogous *p*-nitrophenyl *N*-acetylhexosaminide as substrate.

Competing p-nitrophenyl substrates. Fig. 2 shows Lineweaver & Burk plots for the two separate *p*-nitrophenyl *N*-acetylhexosaminides. From the K_m and V_{max} values derived from these plots the theoretical line was calculated for equimolar mixtures on the assumption of pure competition between these two substrates (Thorn, 1949; Foster & Niemann, 1951). The experimental plot for equimolar mixtures does not agree with this theoretical plot. On the other hand, the total hydrolysis in the mixture is non-additive, so the hydrolysis of the individual substrates is not independent.

Variation of inhibitor concentration. Fig. 3 shows the effect of variation of the concentration of three substances having an inhibitory effect on *N*-acetylglucosaminidase. There is no suggestion of departure from linearity in these plots and therefore no indication that the inhibition is of a partially competitive type (Dixon & Webb, 1958).

Competing phenyl and p-nitrophenyl substrates. Fig. 4 shows the results of experiments from which K_m values were determined for *p*-nitrophenyl *N*-acetylgalactosaminide and phenyl *N*-acetylglucosaminide. At the same time K_i values for each were

Table 4. K_m and V_{max} values

Values of K_m and V_{max} were determined by the method of Lineweaver & Burk (1934). For conditions of assay see text. V_{max} values are expressed as μ moles of substrate hydrolysed/mg. of fresh kidney extracted. Values are given as means $\pm$ S.E. with no. of results in parentheses.

Substrate	Concentration range (mM)	K_m (mM)	V_{max} (μ mole/mg.)
<i>p</i> -Nitrophenyl <i>N</i> -acetylglucosaminide	0.73–5.84	0.72 ± 0.09 (17)	0.156 ± 0.012 (17)
<i>p</i> -Nitrophenyl <i>N</i> -acetylgalactosaminide	0.36–2.56	0.40 ± 0.09 (17)	0.016 ± 0.001 (17)
Phenyl <i>N</i> -acetylglucosaminide	1.00–16.00	1.32 ± 0.13 (7)	0.087 ± 0.012 (7)
Phenyl <i>N</i> -acetylgalactosaminide	1.00–16.00	1.02 ± 0.22 (4)	0.017 ± 0.003 (4)

Table 5. K_i values for various competing substances

K_i values were evaluated by the method of Lineweaver & Burk (1934). The substrates were (a) *p*-nitrophenyl *N*-acetylglucosaminide (0.73–5.84 mM) or (b) *p*-nitrophenyl *N*-acetylgalactosaminide (0.18–1.46 mM). For other conditions see text.

Inhibitors	K_i (mM)	
	(a)	(b)
<i>N</i> -Acetylglucosamine	3.1	7.6
<i>N</i> -Acetylgalactosamine	0.4	1.6
2-Acetamido-2-deoxygluconolactone	0.0007	0.0049
2-Acetamido-2-deoxygalactonolactone	0.0005	0.0018
Competing substrates		
Phenyl <i>N</i> -acetyl- β -D-glucosaminide	0.9	2.6
Phenyl <i>N</i> -acetyl- β -D-galactosaminide	0.2	0.6

Fig. 2. Enzymic hydrolysis of *p*-nitrophenyl *N*-acetylglucosaminide (O), of *p*-nitrophenyl *N*-acetylgalactosaminide (●) and of equimolar mixtures of the two (□). v , Total *p*-nitrophenol liberated (μ g./tube); s , total substrate concn. (mM); ---, theoretical plot for equimolar mixtures on the assumption of pure competition at a single enzyme site. For conditions see text.

Fig. 3. Effect of inhibitor concentration on enzymic hydrolysis. Substrate: *p*-nitrophenyl *N*-acetylglucosaminide (1.45 mM); inhibitors: O, *N*-acetylglucosamine (0.15 M); ●, *N*-acetylgalactosamine (0.045 M). Substrate: phenyl *N*-acetylglucosaminide (8 mM); inhibitor: □, *p*-nitrophenyl *N*-acetylgalactosaminide (0.004 M). v , *p*-Nitrophenol or phenol liberated (μ g./tube); i , inhibitor solution in assay mixture (ml.). For other conditions see text.

determined against the other substrate, by the method of Lineweaver & Burk (1934). From these values and the V_{max} values the theoretical lines were calculated from equations (1) and (2) (Theory section). There is reasonable agreement of these lines with the experimental results obtained with mixtures of these substrates.

Table 6 gives the values of K_m for phenyl *N*-acetylglucosaminide (A) and *p*-nitrophenyl *N*-acetylgalactosaminide (B) and of K_i for these sub-

Table 6. K_m and K_i values and their ratios

	Expt. 1			Expt. 2		
	K_m (mM)	K_i (mM)	K_{mA}/K_{iB}	K_m (mM)	K_i (mM)	K_{iA}/K_{mB}
Phenyl <i>N</i> -acetylglucosaminide (A)	1.1	—	15.7	—	3.5	15.9
<i>p</i> -Nitrophenyl <i>N</i> -acetylgalactosaminide (B)	—	0.07		0.22	—

Fig. 4. Enzymic hydrolysis of phenyl *N*-acetylglucosaminide (O), of *p*-nitrophenyl *N*-acetylgalactosaminide (●) and of mixtures of the two. v , μ -Nitrophenol or phenol liberated (μ moles/tube); (a) concn. (mM) of phenyl *N*-acetylglucosaminide; (b) concn. (mM) of *p*-nitrophenyl *N*-acetylgalactosaminide. □, Liberation of phenol and ■, liberation of *p*-nitrophenol from mixtures containing a ratio (a:b) of 5.5:1. Liberation of phenol (---) and of *p*-nitrophenol (---) from these mixtures calculated according to the text (equations (1) and (2)).

stances when used as inhibitors of the other. The K_i values do not agree with the respective K_m values, but the ratios K_{mA}/K_{iB} and K_{iA}/K_{mB} are equal (see below).

THEORY

The effects on the reaction velocity of varying substrate concentration in the absence and in the presence of a fixed concentration of inhibitor and

of varying inhibitor concentration at fixed substrate concentration resemble formally those given by an enzyme with a single active site and a purely competitive type of inhibitor, as found with a testicular preparation (Woollen *et al.* 1961). With the kidney preparation, however, different K_i values for competitive inhibitors were obtained according to the substrate employed (Table 5). Also the K_m value for *N*-acetylglucosaminide differed from the K_i value when K_i was measured against *N*-acetylgalactosaminide as substrate, and vice versa (Tables 4 and 5). These results cannot be explained on the basis of pure competition at a single site. Two alternative reaction schemes, each of which is compatible with the results, are analysed below. The first permits the conventional formulation of the reactions between enzyme and substrate or inhibitor to be retained but requires that the enzyme has two active sites with special and restricted properties. The second requires only a single active site but includes an additional displacement reaction which permits competition between substrate and inhibitor after, as well as before, combination with the enzyme. It is shown that with competing substrates such a reaction would, in certain circumstances, give rise to the simple quantitative relationship between K_m and K_i values that was observed.

Scheme 1; two specific sites with cross-inhibition. The enzyme has one site at which only *N*-acetylglucosaminides and another at which only *N*-acetylgalactosaminides are broken down. The two sites are completely independent but in a mixture of substrates each substrate acts as a purely competitive inhibitor of the hydrolysis of the other.

Let A and B be the two substrates with concentrations a and b respectively. Let K_{1A} and K_{1B} be the respective Michaelis and inhibitor constants at site 1 and K_{2B} and K_{2A} the respective Michaelis and inhibitor constants at site 2. Let v_A , v_B , V_A , V_B be the initial and the maximum velocities for the enzymic hydrolysis of the two substrates. Then

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{1}{a} \frac{K_{1A}}{V_A} \left(1 + \frac{b}{K_{1B}} \right)$$

and
$$\frac{1}{v_B} = \frac{1}{V_B} + \frac{1}{b} \frac{K_{2B}}{V_B} \left(1 + \frac{a}{K_{2A}} \right).$$

Provided that the hydrolysis of each substrate can be estimated in the presence of the other the values K_{1B} and K_{2A} may be determined by the method of Lineweaver & Burk (1934). If then the rates of hydrolysis of the individual substrates are studied in mixtures of the two, such that $b = xa$, the results may be compared with the theoretically expected rates which are given by equations (1) and (2).

$$\frac{1}{v_A} = \frac{1}{V_A} \left(1 + x \frac{K_{1A}}{K_{1B}} \right) + \frac{1}{a} \frac{K_{1A}}{V_A}, \quad (1)$$

and
$$\frac{1}{v_B} = \frac{1}{V_B} \left(1 + \frac{1}{x} \frac{K_{2B}}{K_{2A}} \right) + \frac{1}{b} \frac{K_{2B}}{V_B}. \quad (2)$$

Scheme 2; single site with displacement. Each substrate combines with the enzyme E at the same active site. When present together the substrates compete for this site and may also displace each other from it after combination with the enzyme. The reactions, with velocity constants and with concentrations given below, may be formulated thus:

If v_A and v_B are the initial velocities of breakdown of EA and EB, V_A and V_B the maximum velocities and the total enzyme

$$e_T = e + p + q \quad (6)$$

then
$$v_A = k_2 p \quad (7)$$

$$v_B = k_4 q \quad (8)$$

$$V_A = k_2 e_T \quad (9)$$

$$V_B = k_4 e_T. \quad (10)$$

The respective Michaelis constants, K_{mA} and K_{mB} , may be measured in the presence of each substrate alone, when

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{1}{a V_A} \frac{k_2 + k_{-1}}{k_1} \quad (11)$$

$$\frac{1}{v_B} = \frac{1}{V_B} + \frac{1}{b V_B} \frac{k_4 + k_{-3}}{k_3} \quad (12)$$

$$K_{mA} = \frac{k_2 + k_{-1}}{k_1} \quad (13)$$

$$K_{mB} = \frac{k_4 + k_{-3}}{k_3}. \quad (14)$$

The solutions of the steady-state equations when both substrates are present is complex. A detailed analysis for a substrate (S) and an inhibitor (I) (Reiner, 1959) shows that the displacement reaction ($ES + I \rightleftharpoons EI + S$) leads in general to a picture which differs from a purely competitive type of inhibition.

However, in the special case of the above scheme which is relevant to the present discussion, i.e. when $k_5 = k_1$ and $k_{-5} = k_3$, then (5) becomes

and, in the steady state, from reactions (3), (4) and (15)

$$\frac{dp}{dt} = 0 = k_1 a(e + q) - p(k_2 + k_{-1} + k_3 b)$$

and
$$\frac{dq}{dt} = 0 = k_3 b(e + p) - q(k_4 + k_{-3} + k_1 a);$$

from which, with equations (6)–(10),

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{1}{a} \frac{1}{V_A} \left(\frac{k_2 + k_{-1} + k_3 b}{k_1} \right) \quad (16)$$

and
$$\frac{1}{v_B} = \frac{1}{V_B} + \frac{1}{b} \frac{1}{V_B} \left(\frac{k_4 + k_{-3} + k_1 a}{k_3} \right) \quad (17)$$

and in an experiment in which a is varied in the absence and in the presence of a fixed concentration b , equations (11) and (16) show that plots of $1/v_A$ versus $1/a$ give the appearance of competitive inhibition with

$$'K_{iB}' = \frac{k_2 + k_{-1}}{k_3}. \quad (18)$$

Similarly, with a fixed concentration a and varying concentration b , equations (12) and (17) would give

$$'K_{iA}' = \frac{k_4 + k_{-3}}{k_1}. \quad (19)$$

From equations (13), (14), (18) and (19)

$$\frac{K_{mA}}{'K_{iB}'} = \frac{'K_{iA}'}{K_{mB}} = \frac{k_3}{k_1} = a \text{ constant}. \quad (20)$$

Such a constant was found experimentally (Table 6). Equations (16) and (17) may be written

$$\frac{1}{v_A} = \frac{1}{V_A} \left(1 + \frac{K_{mA}}{a} \right) + b \frac{k_3}{k_1} \frac{1}{a V_A}$$

and
$$\frac{1}{v_B} = \frac{1}{V_B} \left(1 + \frac{K_{mB}}{b} \right) + a \frac{k_1}{k_3} \frac{1}{b V_B}$$

showing that plots of $1/v_A$ versus b and of $1/v_B$ versus a are straight lines and the inhibition is therefore of the purely competitive type (cf. Fig. 3).

Now, consider mixed substrate experiments in which $b = xa$. From (13) and (16)

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{K_{mA}}{aV_A} + \frac{k_3b}{k_1aV_A}$$

Substituting, $b = xa$,

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{K_{mA}}{aV_A} + \frac{k_3x}{k_1V_A}$$

Substituting for k_3/k_1 , from (20),

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{K_{mA}}{aV_A} + \frac{xK_{mA}}{K_{iB}V_A}$$

$$\text{or} \quad \frac{1}{v_A} = \frac{1}{V_A} \left(1 + \frac{xK_{mA}}{K_{iB}} \right) + \frac{1}{a} \frac{K_{mA}}{V_A} \quad (21)$$

This is the same as (1), so that with mixed-substrate experiments the predicted velocities of breakdown of the individual substrates are the same as those derived from scheme 1.

If, in the scheme described in reactions (3)–(5), the relationship $K_{mA} = K_{mB} \cdot K_D$ holds (where $K_D = k_{-5}/k_5$), either by coincidence or by virtue of $k_2 \ll k_{-1}$ and $k_4 \ll k_{-3}$, the effect of one substrate on the rate of hydrolysis of the other is not affected by the occurrence of the displacement reaction. Such a system cannot be differentiated from the case of simple competition without a displacement reaction.

DISCUSSION

A crude enzyme preparation was used for this work, but experience with partially purified ram-testicular enzyme (Woollen *et al.* 1961) suggests that the difficulties encountered with a purified preparation may well, in future, be overcome by the presence of dilute albumin or Triton X-100 when diluting the purified enzyme.

Attempts to separate the two enzyme activities *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase were as unsuccessful with rat-kidney extract as were those with a preparation from ram testis, and it is concluded that both activities are associated with the same enzyme protein.

In experiments with the two *p*-nitrophenyl substrates both activities were competitively inhibited by *N*-acetylglucosamine, 2-acetamido-2-deoxyglucan-6-phosphate and phenyl *N*-acetyl- β -D-glucosaminide and by the corresponding galactosamine derivatives, but the K_i values for a given inhibitor against each substrate were not equal (Table 5). The experiment with competing substrates confirmed that the mechanism of the reaction was not one of pure competition at a single site (Fig. 2).

Experiments with inhibitors at various concentrations (Fig. 3) gave no indication that the com-

petition was of a partial rather than a pure type (Dixon & Webb, 1958).

Two theoretical reaction schemes which meet the requirements of these results were analysed. Both led to identical predicted velocities of breakdown in mixtures of two substrates which were in reasonable agreement with the experimental results (Fig. 4). However, the second scheme, which postulated a single active site at which a displacement mechanism operated in addition to the usual competition, also required a relationship between the Michaelis and inhibitor constants for competing substrates. This relationship was observed experimentally.

The restriction on the values of the rate constants which was introduced in deriving the above scheme requires the rate of formation of enzyme-substrate complex to be the same whether the substrate reacts with the free enzyme or displaces another substrate from its complex with the enzyme. This requirement may be less improbable than appears at first sight. The reactions may be considerably more complex than those formulated, which involve only single intermediate enzyme-substrate complexes, and it is possible that the reaction between free enzyme and substrate and the displacement reaction share a common rate-limiting step.

For the particular scheme of displacement suggested here it is necessary to measure Michaelis and inhibitor constants of two competing substrates. Reiner (1959) shows the circumstances in which a general inhibitor mechanism involving displacement gives different experimental results from other inhibitor mechanisms. It may well be that these mechanisms involving displacement may be more commonly found by suitable analysis of enzyme systems where Michaelis and inhibitor constants of substrates have been at variance.

SUMMARY

1. Two *N*-acetylhexosaminidase activities of extracts of rat kidney have been measured with the substrates *p*-nitrophenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide.

2. No separation of the activities was achieved by fractionation with ammonium sulphate or with calcium phosphate gel or by partial heat inactivation.

3. The K_i values of six competing substances, *N*-acetylglucosamine, 2-acetamido-2-deoxyglucan-6-phosphate, phenyl *N*-acetylglucosaminide and the corresponding galactosamine derivatives were not the same when measured against *p*-nitrophenyl *N*-acetylglucosaminide and *N*-acetylgalactosaminide respectively.

4. There was no evidence of partially competitive inhibition.

5. Two schemes are suggested for the enzyme mechanism. The first is of two independent sites for the two activities on the same enzyme protein, together with inhibition of *N*-acetylglucosaminidase by *N*-acetylgalactosaminides and inhibition of *N*-acetylgalactosaminidase by *N*-acetylglucosaminides. The second is of one site with competition, together with a direct displacement mechanism of one substrate by another with special assumptions for the velocity constants for the displacement.

6. Further experimental evidence of a relationship between Michaelis and inhibitor constants of two competing substrates supports the second scheme.

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Studies on Glucosaminidase

6. *N*-ACETYL- β -GLUCOSAMINIDASE AND *N*-ACETYL- β -GALACTOSAMINIDASE ACTIVITIES OF A VARIETY OF ENZYME PREPARATIONS

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A survey of a large number of enzyme preparations for *N*-acetyl- β -D-glucosaminidase and *N*-acetyl- β -D-galactosaminidase activities has been undertaken to establish whether the close association of these activities which had been found with a testicular preparation (Woollen, Heyworth & Walker, 1961) and a kidney preparation (Walker, Woollen & Heyworth, 1961) was general. No enzyme source was discovered which had one activity and not the other. The two activities were never additive when both substrates were present together, so there was no evidence of specific sites.

MATERIALS AND METHODS

Enzyme preparations

Human pregnancy sera. These were obtained from one subject at 6 and 24 weeks of pregnancy.

Pig epididymis. Crude and purified preparations corresponding to the 'best bit', stages 2 and 7b, of specific activity 194 000 and 3 280 000 (Findlay & Levvy, 1960) were diluted in 0.1% (w/v) crystallized bovine albumin (Armour Ltd.) for assay.

Ram testis. The freeze-dried extract (Benger Ltd., Holmes Chapel, Cheshire) was made 0.1% (w/v) in 0.1% (w/v) crystallized bovine albumin (Woollen *et al.* 1961).

Rat tissues. Fresh tissues were weighed and ground in water in a glass homogenizer and made to a final concentration (w/v) of 10% for liver, spleen and testis, 2% for kidney and salivary gland (submaxillary and sublingual) and 1% for epididymis, in 0.05M-sodium citrate-citric acid buffer, pH 4.5. Clear supernatants were obtained by centrifuging at 1500g for 10 min. Such supernatants have 80-90% of the *N*-acetylglucosaminidase activity of whole homogenates.

Viper venom. Russell viper venom (Wellcome Research Laboratories), 1% (w/v) in water, was used.

Leech. *Hirudo medicinalis* specimens were killed with chloroform, and were weighed and homogenized in water. The extract (10%, w/v) was centrifuged at 1500g for 10 min. to obtain a clear supernatant. A 5% (w/v) extract of leech heads was similarly prepared.

Snail and limpet preparations. The digestive tract of *Helix pomatia* and the digestive gland of *Patella vulgata* (both killed with chloroform) were weighed, homogenized with water and diluted to give concentrations (w/v) of 4% and 10% respectively.