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.

Locust-crop fluid. This was obtained from adult *Schistocerca gregaria* at the Anti-Locust Research Centre, London.

Sweet-almond emulsin. Partially de-fatted sweet-almond meal (5 g.) was shaken at 4° with 100 ml. of 0.05 M-citrate buffer, pH 4.5, for 15 min. and then allowed to stand for 2 hr. at 4° and filtered to give a clear extract.

Micro-organisms

Aspergillus oryzae. This preparation was a 0.1% (w/v) solution in water of Hydrolase Mixture (C. F. Boehringer und Söhne, Mannheim, Germany).

Proteus vulgaris. A freeze-dried extract (Dodgson, Lloyd & Spencer, 1957; preparation B) was made 1% (w/v) in water.

Trichomonas foetus. A freeze-dried extract (Watkins, 1959) was made 0.05% (w/v) in 1% (w/v) crystallized bovine albumin.

Lycoperdon pyriforma and *Lycoperdon giganteum* extracts. These were prepared from the fungi according to Tracey (1955).

Clostridium perfringens (N.C.T.C. 2836). A 10 hr.

culture in Robertson's cooked-meat medium (see Mackie & McCartney, 1942) was subcultured into 250 ml. of the same medium and incubated for 12 hr. in screw-capped bottles at 37°. The cultures were filtered through muslin to remove meat particles, centrifuged at 14 000g for 30 min., resuspended in 0.9% sodium chloride and again centrifuged. The cells were disrupted with polishing γ -alumina (McIlwain, 1948) and the alumina was eluted with 0.1 M-sodium phosphate buffer, pH 7.4, containing 10% (w/v) of ammonium sulphate. This solution was dialysed against 1% (w/v) sodium chloride for 48 hr. and, for some investigations, made 1% (w/v) with crystallized bovine albumin (see Results section).

Cultures were grown at 37° for 16 hr. of *Alcaligenes faecalis*, *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Staphylococcus pyogenes* and *Enterococcus* on digest broth; of *Escherichia coli* in peptone water; of *Streptococcus pyogenes* (Lancefield group A) in glucose broth; of *Clostridium sporogenes* in Robertson's cooked-meat medium; and for 48 hr. of *Pneumococcus* type 3 in horse-serum broth. After centrifuging the cells were suspended in 10 ml. of 0.9% sodium chloride.

Table 1. *N-Acetylglucosaminidase and N-acetylgalactosaminidase activities*

Substrates were 1.75 mm-*p*-nitrophenyl *N*-acetylglucosaminide or 1.75 mm-*p*-nitrophenyl *N*-acetylgalactosaminide or both. For other conditions see the Methods section. Activities are expressed as μ g. of *p*-nitrophenol liberated/unit of enzyme source indicated.

Substrates	Activities			Units of enzyme source
	<i>N</i> -Acetylglucosaminide	<i>N</i> -Acetylgalactosaminide	<i>N</i> -Acetylglucosaminide + <i>N</i> -acetylgalactosaminide	
Enzyme preparations:				
Human pregnancy serum*				
6 weeks	47	6	14	ml.
24 weeks	159	18	44	ml.
Pig epididymis				
Stage 2	0.31 $\times 10^6$	0.05 $\times 10^6$	0.12 $\times 10^6$	ml.
Stage 7 b	23.10 $\times 10^6$	3.75 $\times 10^6$	7.95 $\times 10^6$	ml.
Ram testis	314	68	144	mg.
Rat-tissue extracts				
Kidney	16.0	1.9	4.0	mg.
Liver	5.3	0.8	1.9	mg.
Spleen	4.8	0.8	1.5	mg.
Testis	2.8	0.4	1.1	mg.
Salivary gland	22.8	2.3	5.1	mg.
Epididymis	103.8	10.8	25.2	mg.
Viper venom	19.3	2.9	5.6	mg.
<i>Hirudo medicinalis</i>				
Whole	19.4	10.5	13.7	mg.
Head	15.5	7.1	9.5	mg.
<i>Helix pomatia</i> digestive tract	72.7	33.1	56.0	mg.
<i>Patella vulgata</i> digestive gland	13.4	5.4	15.1	mg.
<i>Schistocerca gregaria</i> crop fluid	64.6 $\times 10^3$	26.0 $\times 10^3$	40.8 $\times 10^3$	ml.
Sweet-almond emulsin	8.2	5.4	5.6	mg.
<i>Aspergillus oryzae</i>	260	221	214	mg.
<i>Proteus vulgaris</i>	31.4	4.7	10.3	mg.
<i>Trichomonas foetus</i>	472	352	414	mg.
<i>Lycoperdon pyriforma</i>	460	320	410	ml.
<i>L. giganteum</i>	520	790	460	ml.
<i>Clostridium perfringens</i> †	224	12	216	ml.
<i>Pneumococcus</i> , type 3	348	45	325	ml.
<i>Enterococcus</i>	180	7	172	ml.

* At pH 4.3 (Walker *et al.* 1960).

† At pH 5.05

Substrates

p-Nitrophenyl *N*-acetyl- β -D-glucosaminide and the corresponding galactosaminide were prepared according to Leaback & Walker (1957) and Heyworth, Leaback & Walker (1959) respectively.

Estimation of enzyme activities

The method is described by Woollen *et al.* (1961). Except where indicated, the enzyme solutions were incubated with substrate(s) in a total volume of 1 ml. for 30 min. in 0.05M-citrate buffer, pH 4.5, at 37°.

Antitoxin

The *C. perfringens* type A antiserum was from The Wellcome Research Laboratories (reference EX 1863).

RESULTS

N-Acetylglucosaminidase and *N*-acetylgalactosaminidase activities of the various enzyme preparations. The amount of hydrolysis was measured in the presence of 1.75 mM-*p*-nitrophenyl *N*-acetylglucosaminide or 1.75 mM-*p*-nitrophenyl *N*-acetylgalactosaminide or in mixtures containing these concentrations of both substrates (Table 1). *N*-Acetylglucosaminidase activity was always accompanied by *N*-acetylgalactosaminidase activity, although there was a wide variation in the ratio of these activities (1–26) in the different sources under the conditions used. There was no apparent correlation between the ratios and the biological origins of the enzyme preparations.

With human pregnancy serum the *N*-acetylgalactosaminidase activity appeared to be increasing during pregnancy parallel with *N*-acetylglucosaminidase (Walker, Woollen & Pugh, 1960). Similarly, with the purified preparations of pig epididymis (Findlay & Levvy, 1960), the relative *N*-acetylglucosaminidase activity to *N*-acetylgalactosaminidase activity remained constant over the 17-fold increase in specific activity.

The relative *N*-acetylglucosaminidase activities

of the various rat tissues are in agreement with those found by Conchie, Findlay & Levvy (1959) with whole homogenates.

The *N*-acetylgalactosaminidase activity of *T. foetus* was greater, relative to *N*-acetylglucosaminidase, than that found at pH 7 by Watkins (1959).

In every case the amount of hydrolysis in mixtures of the two substrates was less than the sum of those obtained with each substrate individually.

No activity was detected with either substrate under the conditions used with *P. aeruginosa*, *C. sporogenes*, *Alcaligenes faecalis*, *B. megaterium*, *Strep. pyogenes*, *Staphylococcus pyrogenes* or *E. coli*.

Fig. 1. Effect of pH on the emulsin activities. For 3.6 mM-*p*-nitrophenyl *N*-acetylglucosaminide and 0.1% (w/v) of enzyme (O) a relative activity of 100 corresponds to 10.5 μ g. of *p*-nitrophenol liberated/tube. For 2.2 mM-*p*-nitrophenyl *N*-acetylgalactosaminide and 0.3% (w/v) of enzyme (●) a relative activity of 100 corresponds to 23.3 μ g. of *p*-nitrophenol liberated/tube. For other conditions see the Methods section.

Table 2. Ammonium sulphate fractionation and partial heat inactivation of emulsin

Fractions were precipitated between the indicated limits of percentage saturation with ammonium sulphate. Preincubation was carried out at 56° in the absence of substrate in 0.05M-citrate buffer, pH 4.5. For the assays the substrate concentration was 2.2 mM. For other conditions see the Methods section.

Period of preincubation (min.) ...	N-Acetylglucosaminidase (% of untreated preparation)		Ratio N-acetylglucosaminidase: N-acetylgalactosaminidase	
	0	5	0	5
Enzyme preparation:				
Untreated	100*	66.0	1.7	2.4
(NH ₄) ₂ SO ₄ fractions				
0–40 %	4.3	2.3	1.2	1.9
40–50 %	4.4	2.4	1.1	2.0
50–60 %	40.1	26.4	1.7	2.3

* 60.4 μ g. of *p*-nitrophenol liberated/tube.

N-Acetylhexosaminidase activities of sweet-almond emulsin. A kinetic study of the two activities in emulsin was initiated like the studies on a testicular preparation (Woollen *et al.* 1961) and a kidney extract (Walker *et al.* 1961). The effect of pH was studied in 0.05M-citrate buffer (Fig. 1). At pH 4.5 both enzyme activities were proportional to enzyme concentration over a fourfold range and also to time of incubation over 1 hr. Although substrate competition was demonstrated, further experiments showed differences from the testis and kidney preparations. Fractionation with ammonium sulphate and partial heat inactivation of these fractions as described by Woollen *et al.* (1961) both led to variations in the ratio of *N*-acetylglucosaminidase:*N*-acetylgalactosaminidase activities (Table 2). This may indicate the presence of two or more enzyme proteins of unknown specificity.

N-Acetylhexosaminidase activity of *Clostridium perfringens*. An initial inability to detect *N*-acetylgalactosaminidase activity in these preparations led to an investigation of optimum conditions. Experience with a testicular preparation had shown instability of *N*-acetylglucosaminidase and a loss of specific activity on dilution of the partially purified enzyme, both of which were overcome in the presence of 0.1% (w/v) crystallized bovine albumin. A similar loss of specific activity on dilution was observed with the *C. perfringens* pre-

paration. The presence of albumin was found to increase the rate of hydrolysis of *p*-nitrophenyl-*N*-acetylglucosaminide (Fig. 2) and in a final concentration of 0.1% (w/v) gave proportionality between activity and enzyme concentration. Activity was also proportional to time of incubation over 45 min. In the presence of 0.1% of albumin there was an optimum pH of 5.05 in 0.05M-citrate buffer (Fig. 3) and *N*-acetylgalactosaminidase activity was found under these optimum conditions (Table 1). Under these conditions *C. perfringens* antitoxin was shown to be a non-competitive inhibitor of *N*-acetylglucosaminidase activity.

DISCUSSION

It was concluded from the failure of all attempts to separate the *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities in ram testis (Woollen *et al.* 1961) and rat kidney (Walker *et al.* 1961) and from the detailed kinetic studies carried out with those preparations that both activities were associated with a single active site on the same enzyme protein. In the present paper it was shown that every enzyme preparation that had *N*-acetyl-

Fig. 2. Effect of albumin concentration on *C. perfringens* *N*-acetylglucosaminidase activity in 0.05M-citrate buffer, pH 5.05, with 3.6 mM-*p*-nitrophenyl *N*-acetylglucosaminide. For other conditions see the Methods section.

Fig. 3. Effect of pH on *N*-acetylglucosaminidase activity of *C. perfringens* with 0.05M-citrate buffer and 3.6 mM-*p*-nitrophenyl *N*-acetylglucosaminide. For other conditions see the Methods section. A relative activity of 100 corresponds to 51.8 µg. of *p*-nitrophenol liberated/tube.

glucosaminidase activity also had *N*-acetylgalactosaminidase activity and that in each case the total hydrolysis in mixtures of the two substrates was less than the sum of those of the individual substrates. There was therefore no evidence of a specific site for either substrate.

It cannot, however, be concluded from these results that the same enzyme is responsible for the hydrolysis of both substrates in every case. Fractionation and differential inactivation of the activities in emulsin indicated the presence of more than one active protein. More detailed investigations are required before any further conclusion can be drawn about the relationship of *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activity in each case.

The increased *N*-acetylglucosaminidase activity in the *C. perfringens* enzyme preparation in the presence of albumin appears to be another example of protection by surface-active agents against inactivation in purified enzymes. This has been described for *N*-acetylglucosaminidase (Woollen *et al.* 1961), for β -glucuronidase (Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954), for acid phosphatase (Tsuboi & Hudson, 1955) and cellulase (Myers & Northcote, 1959).

C. perfringens antitoxin has been shown to inhibit the hyaluronidase (McClean & Hale, 1941) and the lecithinase (Macfarlane & Knight, 1941) of this organism, the latter non-competitively (Cinader, 1953). The antitoxin has been shown here to act non-competitively with *N*-acetylglucosaminidase.

SUMMARY

1. The *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities of a wide variety of enzyme preparations have been measured.

2. Where *N*-acetylglucosaminidase was detected it was accompanied by *N*-acetylgalactosaminidase but the ratios of the two activities covered a wide range.

3. Experiments with mixed substrates did not indicate a specific site for either activity in any of the preparations.

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The Metabolism of Protocatechuic Acid by a *Vibrio*

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Recent work with micro-organisms has suggested that the mechanisms of biological ring-fission may be considerably more diverse than was originally suspected. Not only has a new mode of fission of the benzene nucleus been reported

(Dagley & Stopher, 1959; Trippett, Stopher & Dagley, 1960), but additional pathways have been formulated whereby the aliphatic products resulting from ring-fission are channelled into terminal oxidation cycles (Ribbons & Evans, 1959). This paper describes the metabolism of protocatechuic acid by *Vibrio* O1 (Happold & Key, 1932)

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