CXCIV. THE HYDROLYSIS OF GLUCOSAMINIDES BY AN ENZYME IN *HELIX POMATIA*

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It has long been known that the polysaccharide chitin when completely hydrolysed yields glucosamine and acetic acid; in recent years it has indeed been shown conclusively that the basic unit of the polysaccharide is *N*-acetylglucosamine. The evidence for the latter statement rests, firstly, on the discovery by Karrer & Hofmann [1929] of an enzyme in *Helix pomatia* which is able to hydrolyse chitin to *N*-acetylglucosamine and, secondly, on the isolation of chitobiose octaacetate by Bergmann *et al.* [1931] and by Zechmeister & Toth [1931] as a product of acetolysis of chitin.

In view of the conclusions which have been reached concerning the structure of polysaccharides from experiments on their hydrolysis by enzymes possessing α - or β -specificity, it was thought that an enzyme such as that discovered by Karrer might, if it could be characterized, be used to investigate the stereochemical structure not only of chitin itself but of other glucosamine-containing polysaccharides such as that of egg albumin [Neuberger, 1938]. For this purpose it was clearly necessary to determine the α - or β -specificity of the enzyme before any conclusions could be reached as to the configuration of the natural linkages in such polysaccharides, and it was also thought desirable to investigate other factors which might influence enzyme action, such as variations in the *N*-acyl group and substitution on carbon atoms other than C₁.

Up to the present, little has been known about the specificity of chitin-splitting enzymes. Zechmeister and his co-workers [1932] found that emulsin contains an enzyme capable of hydrolysing chitodextrin, a product obtained by partial acid hydrolysis of chitin. It was first presumed by these authors that the enzyme responsible for hydrolysis was the glucosidase in emulsin which is known to possess β -specificity, and they therefore concluded that chitodextrin and chitin have a β -structure. The assumption they made was shown to be incorrect by Helferich & Iloff [1933], who found that β -glucosidase and chitinase were different enzymes. Later, Zechmeister and his co-workers [1934] confirmed Helferich's observation, by demonstrating that a highly purified sample of β -glucosidase is inactive towards chitin.

With regard to the *N*-acyl specificity of the enzyme, some preliminary experiments were done by Karrer & White [1930]; these authors treated chitin with strong alkali and obtained a poorly characterized but presumably partly deacetylated product, chitosan, which, on enzymic hydrolysis, yielded a mixture. If, however, the chitosan were reacylated, the product of enzymic hydrolysis was pure *N*-acetylglucosamine. Products purporting to be *N*-formyl, *N*-propionyl, *N*-butyryl and *N*-benzoyl derivatives of chitosan were also prepared, of which the only one to be attacked by the enzyme was the *N*-benzoyl derivative, and that to only a small extent. These experiments suggest that the enzyme will

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not attack linkages between free glucosamine residues and is indeed specific towards *N*-acetyl compounds, but the matter cannot be regarded as settled; no analytical evidence bearing on the composition of the reacylated derivatives was presented and in so far at least as the formyl derivative is concerned, Karrer's conditions of formylation were not such as, in our experience, would lead to any acylation.

Further work on the *N*-acyl specificity of this type of enzyme was done by Helferich *et al.* [1934], who examined the action of emulsin on phenyl glucosaminides. They found that emulsin contains an enzyme which hydrolyses *N*-acetyl-phenylglucosaminide, although it will not hydrolyse the corresponding unacylated compound; this latter observation is in agreement with that of Irvine & Hynd [1939] who found that emulsin cannot hydrolyse methylglucosaminide hydrochloride. This experiment provides further evidence that the enzyme will only hydrolyse acylated glucosamine derivatives. As regards the configuration of Helferich's phenylglucosaminide, the method of preparation and the rotation indicate that it has a β -configuration, but since no corresponding α -compound was available for purposes of comparison, this matter cannot be decided with any certainty.

Since a pair of *N*-acetyl methylglucosaminides were prepared by the authors [1939] it became possible to discover whether one of the enzymes capable of hydrolysing glucosaminides showed either α - or β -specificity, and the *Helix pomatia* enzyme was chosen for this purpose. In order to investigate the *N*-acyl specificity of the enzyme, various *N*-acylated glucosaminides were prepared, and the effect of the enzyme on these, as well as on *O*-substituted glucosaminides already described in the literature, was examined.

EXPERIMENTAL

(1) Preparation of compounds

N-*p*-Toluenesulphonyl tetra-acetylglucosamine. Tetra-acetylglucosamine was prepared according to Bergmann & Zervas [1931] from 3.83 g. of tetra-acetylglucosamine hydrochloride, and dissolved in 20 ml. dry pyridine. *p*-Toluenesulphonyl chloride (1.9 g.), recrystallized from ligroin, was dissolved in pyridine and the two solutions were mixed, left at room temperature for 2½ hr. and heated for 30 min. on a boiling water bath; the solution was then poured into iced water and extracted with chloroform. The chloroform solution was washed with *N* HCl and water, dried over sodium sulphate and evaporated to dryness under diminished pressure. The substance crystallized from 30 % methyl alcohol in needles. Yield 56 %. M.P. 128–129°. $[\alpha]_D - 3^\circ$ in chloroform. (Found: N, 2.8 %; $C_{21}H_{27}O_{11}NS$ requires N, 2.8 %.)

1: *Bromo-N-p-toluenesulphonyl triacetylglucosamine*. *N-p*-Toluenesulphonyl tetra-acetylglucosamine (1 g.) was dissolved in glacial acetic acid (10 ml.) saturated at 0° with HBr. The solution was cooled to 0° and HBr was passed in for 45 min. It was then left for 2 hr. at room temperature, poured on to crushed ice and extracted with chloroform. The chloroform solution was washed with ice-cold bicarbonate solution till neutral to litmus, and once with brine, and dried over sodium sulphate. The chloroform was removed under diminished pressure and finally *in vacuo*. Crude yield 83 %. The compound crystallized from glacial acetic acid and dry ether in needles. M.P. 148°. $[\alpha]_D + 63.5$ (in chloroform). (Found: C, 43.74; H, 4.83; Br, 15.2 %. $C_{19}H_{24}O_9NSBr$ requires C, 43.70; H, 4.60; Br, 15.33 %.)

N-p-Toluenesulphonyl triacetylphenylglucosaminide. Sodium (0.77 g.) was dissolved in dry phenol (18.2 g.) and 1-bromo-*N-p*-toluenesulphonyl triacetylglucosamine (1.86 g.) was added. The solution was heated on a water bath with exclusion of moisture for 1 hr. with continuous shaking, and poured into cold water. The oil presently solidified; it was washed several times with cold water, and filtered. The compound crystallized from absolute alcohol in needles. Yield 1.5 g. m.p. 200–201°. $[\alpha]_D - 52.8^\circ$ in pyridine. (Found: N, 2.7%; $C_{25}H_{29}O_{10}NS$ requires N, 2.6%.)

N-p-Toluenesulphonyl phenylglucosaminide. *N-p*-Toluenesulphonyl triacetyl β -phenylglucosaminide (0.535 g.; 1 millimol.) was dissolved in 10 ml. absolute methyl alcohol, 0.5 millimol. barium methoxide was added and the mixture was left at room temperature for 2 hr. The solution was exactly neutralized with 5*N* H_2SO_4 and filtered. The barium sulphate was extracted twice with absolute alcohol and the combined solutions were evaporated to a low volume and cooled. Yield 91%. The compound crystallized from absolute alcohol in needles. m.p. 213–214°. $[\alpha]_D - 83^\circ$ in pyridine. (Found: C, 55.57; H, 5.705; N, 3.43%. $C_{19}H_{27}O_7NS$ requires C, 55.61; H, 5.854; N, 3.4%.)

It was desired to obtain an *N*-formylglucosaminide, and attempts were made to prepare both β -methyl and β -phenyl triacetyl *N*-formylglucosaminides by heating the corresponding free bases with formic acid distilled under diminished pressure from anhydrous copper sulphate. No formyl compound was obtained. It was found, however, that if β -methyl triacetylglucosaminide formate is heated to 155° for 3 hr. the pure formyl compound is obtained in very good yield.

N-Carbobenzyloxy triacetyl- β -methylglucosaminide [Neuberger & Pitt Rivers, 1939]. *N*-Carbobenzyloxy- β -methylglucosaminide (1 g.) was dissolved in dry pyridine (10 ml.) and dry acetic anhydride (8 ml.) was added. The mixture was left for 14 hr. at 2°, poured on to crushed ice, filtered and washed with ice-cold water. The substance crystallized from 30% methyl alcohol in large needles. Yield 1.2 g. (87%). m.p. 147–149°. $[\alpha]_D + 15^\circ$ in chloroform. (Found: N, 3.09%. $C_{21}H_{28}ON$ requires N, 3.08%.)

Triacetyl- β -methylglucosaminide formate. *N*-Carbobenzyloxy triacetyl- β -methylglucosaminide (0.5 g.) was dissolved in 15 ml. dry formic acid and catalytically reduced in the presence of Pd black. The formic acid was removed under diminished pressure, chloroform was added and the compound isolated by precipitation with ether. Yield 0.3 g. (75%). m.p. 120°. 18.2 mg. of this compound required 2.5 ml. *N*/50 NaOH using bromothymol blue as indicator (calculated, 2.49 ml.).

N-Formyl triacetyl- β -methylglucosaminide. Triacetyl- β -methylglucosaminide formate (0.5 g.) was heated to 155° in boiling anisole for 3 hr. The anisole was removed under diminished pressure at 60°. Yield 0.25 g. m.p. 165°. (Found: N, 4.12%. $C_{14}H_{21}O_6N$ requires N, 4.03%.)

N-Formyl- β -methylglucosaminide. *N*-Formyltriacetyl- β -methylglucosaminide (0.25 g.) was dissolved in 0.2 ml. water and treated with 3 equiv. of aqueous baryta. The solution was left at room temperature for 2 hr., exactly neutralized with H_2SO_4 and centrifuged. The water was removed under diminished pressure at 35°. Alcohol was added several times and evaporated off. The substance crystallized from absolute alcohol and ether in needles. Yield 0.149 g. (93%). m.p. 204–205°. $[\alpha]_D - 47.2^\circ$ in water. (Found: C, 43.17; H, 7.58; N, 6.2%. $C_8H_{15}O_6N$ requires C, 43.43; H, 6.8; N, 6.33%.)

It will be observed that the analysis of this compound is unsatisfactory in respect of the hydrogen; a similarly high figure for the hydrogen was obtained by an independent analyst. In spite of this, however, we feel no doubt concerning

the identity of the substance in view of the following experiments: (a) hydrolysis of a small amount with 5*N* HCl for 20 hr. gave an almost theoretical yield of glucosamine hydrochloride having $[\alpha]_D + 73^\circ$ (equilibrium value in water) and N 6.3%; (b) analysis by the method of Pregl & Soltys [1929] gave volatile acid amounting to 13.09% calculated as formic acid (theory 13.1%); moreover the distillate after neutralization and concentration reduced ammoniacal silver nitrate on warming.

N-Propionyl-triacetyl- β -phenylglucosaminide. Triacetyl- β -phenylglucosaminide hydrochloride (0.417 g.) was suspended in chloroform (20 ml.); the suspension was cooled in ice and triethylamine (0.32 ml.) was added. Propionyl chloride (0.12 ml.) was added and the solution left at room temperature for 1 hr. Chloroform (20 ml.) was added and the chloroform solution was washed successively with *N* HCl, bicarbonate solution and water, and dried over sodium sulphate. The chloroform was removed under diminished pressure. Yield 78%. The residue crystallized from 50% alcohol in needles. m.p. 197–197.5°. $[\alpha]_D - 10^\circ$ in chloroform. (Found: N, 3.1%. $C_{21}H_{27}O_9N$ requires N, 3.2%.)

N-Propionyl β -phenylglucosaminide. *N*-Propionyl triacetyl- β -phenylglucosaminide (0.56 g.) was dissolved in methyl alcohol (10 ml.), and *N* sodium methoxide (3 ml.) was added. The solution was left at room temperature for 2 hr., and exactly neutralized with 2*N* H_2SO_4 . The solution was filtered and the sodium sulphate was extracted several times with boiling alcohol. The combined extracts and filtrate were evaporated to dryness, re-extracted five times with hot alcohol and evaporated to a low volume from which the compound crystallized in needles. Yield 0.38 g. (95%). m.p. 230° with decomp. $[\alpha]_D + 8^\circ$ in pyridine. (Found: C, 57.73; H, 6.78; N, 4.44%. $C_{13}H_{21}O_6N$ requires C, 57.9; H, 6.75; N, 4.5%.)

N-Butyryl triacetyl- β -phenylglucosaminide. This compound was prepared from triacetyl- β -phenylglucosaminide and butyryl chloride in the same way as the propionyl compound. Yield 0.33 g. The substance crystallized from aqueous alcohol in needles. m.p. 178–179°. $[\alpha]_D - 10^\circ$ in chloroform. (Found: N, 3.1%. $C_{22}H_{29}O_9N$ requires N, 3.1%.)

(2) Enzyme experiments

Preparation of the enzyme solution from Helix pomatia. Fifteen snails were dissected, and the whole of the digestive tract and liver were removed. The organs were ground with clean sand and extracted with 100 ml. A.R. glycerol. The glycerol extract was filtered through muslin, and the sand and macerate were re-extracted twice with 25 ml. glycerol and filtered. Solid matter was removed by centrifuging, and the glycerol extract was dialysed for 16 hr. against running tap water and for 2 hr. against distilled water. It was necessary to use a bladder for dialysis, since the cellulase in the extract destroyed cellophane membranes. The aqueous extract (250 ml.) after dialysis was kept at 2°, and retained its activity with respect to β -glucosidase and glucosaminidase after 4 months. The cellulase activity disappeared in time. When the solution was first prepared it was opaque, and no solid matter could be removed by centrifuging, but it cleared over a period of 3 months, a light brown precipitate settling in the bottom of the flask, and eventually became translucent.

Methods of determining enzyme activity. Two methods were used. For methylglucosaminides and polysaccharides, the reducing sugar liberated by hydrolysis of the glycoside link was estimated by Hanes' [1929] modification of the method of Hagedorn & Jensen. It was found by Moggridge & Neuberger [1938] that the amount of thiosulphate needed was strictly proportional to the amount of *N*-acetylglucosamine present, 2.48 ml. of *N*/100 thiosulphate being equivalent to

1 mg. of *N*-acetylglucosamine. This method was not applicable in the case of phenylglucosaminides, since the phenol liberated also takes up iodine but not in stoichiometric proportions. For these experiments, phenol determinations by the method of Folin & Ciocalteu [1927] were done. β -Glucosidase activity was measured by the rate of hydrolysis of salicin, the saligenin liberated being measured by the method of Folin & Ciocalteu [1927].

The samples to be estimated were treated as follows: 2 ml. were removed from the hydrolysis solution and 3 drops of 5 % sodium bicarbonate solution and 1 ml. of dialysed iron (the stock solution B.P. was diluted ten times) were added. The solution was filtered and washed with water till the volume was 10 ml. An aliquot was taken and estimated by one of the methods given.

Effect of pH on N-acetylglucosaminidase. The pH range from 1.5 to 9.0 was covered by buffers made up as follows: the lowest points were obtained by adding suitable amounts of HCl to the reaction mixture; the range from pH 3 to 6 was covered by phthalate and acetate buffers. This was done in order to determine whether alteration in the anion produced any effect on the activity of the enzyme. The higher pH range was covered by phosphate and borate buffers. Tubes containing 10 mg. of β -phenyl *N*-acetylglucosaminide, 0.3 ml. of enzyme solution, 0.2 ml. of buffer and 1.5 ml. water were left for 5 hr. at 38°, and the amount of phenol liberated was determined colorimetrically by the method of Folin & Ciocalteu [1927]. Control experiments were done on the substrate and buffer from pH 4 downwards, since it was expected that acid hydrolysis of the glucosaminide might be significant; actually, however, even at pH 2.5 less than 0.5 % of phenol was liberated by acid hydrolysis. The true pH was measured, using a capillator, since it was found that the enzyme solution itself exerts a buffering action. The results are given in Table I.

Table I

	pH	% splitting
HCl	2.6	25.2
	3.2	66.0
Phthalate	3.3	68.0
	3.6	71.0
	4.0	65.0
	4.5	56.0
	5.0	43.0
	5.5	35.6
Acetate	6.0	27.6
	4.0	63.6
	4.5	59.8
	5.0	45.3
Phosphate	5.5	34.1
	6.5	23.4
	7.0	21.7
Borate	9.0	9.9

It appears, therefore, that within the limits of experimental error, the anion of the buffer has no effect on the enzyme. The pH optimum for the β -glucosaminidase is 3.6.

Kinetics of the enzymic hydrolysis. For purposes of standardization, it was considered important to investigate the time relationship of enzymic hydrolysis. 50 mg. of β -phenyl *N*-acetylglucosamine were dissolved in 15 ml. of water; 2.5 ml. of phthalate buffer, pH 4.0, and 2.5 ml. of enzyme solution were added. The reaction mixture was kept at 38°, and 2 ml. portions were removed at short intervals. The results are given in Table II. *k* is calculated as a constant for a unimolecular reaction.

Table II

Time (min.)	% splitting	<i>k</i>
20	21.8	0.00123
40	34.2	0.00104
60	46.3	0.00103
80	57.2	0.00106
100	64.6	0.001035
120	70.0	0.00100
160	79.6	0.00099
200	82.2	0.00086
270	83.8	0.00067

From Table II it appears that, up to about 65% hydrolysis, the reaction follows a unimolecular course. Afterwards, there is an increasing diminution of *k*, a phenomenon which has been often observed in enzymic reactions.

By extrapolation of the data given in Table II, it was found that the half-time value for this concentration of enzyme and substrate was 75 min. This experiment was repeated under almost identical conditions after an interval of 12 weeks, and the half-time value was found to be the same.

Effect of temperature on the Helix pomatia glucosaminidase. Samples containing 5 mg. of β -phenyl *N*-acetylglucosaminide, dissolved in 1.5 ml. water, with 0.25 ml. phthalate buffer (*pH* 4.0) and 0.25 ml. enzyme solution, were left at temperatures of 0, 9, 17.5, 25, 37 and 54.4°, and the amount of hydrolysis after 1.5 hr. was determined.

Table III

Temperature (°C.)	% splitting
0	13.1
9	23.6
17.5	39.3
25	54.7
37.5	61.7
54.5	39.3

It appears that the optimum temperature for this enzyme is at 37.5°. The activity of the enzyme is completely destroyed if the solution is boiled for 30 sec.

Action of Helix pomatia glucosaminidase on N-acetylmethylglucosaminides. Small test tubes containing 4 mg. of *N*-acetyl methylglucosaminide, 0.1 ml. of *M*/10 phthalate buffer (*pH* 3.5) and 0.1 ml. of enzyme solution were made up to 2 ml. with distilled water and 2 drops of toluene were added. The tubes were removed at measured intervals of time, the amount of splitting being measured by Hanes' method. Measurement at zero time showed that the enzyme solution has an initial reducing value, but a control experiment carried out at 38° on the enzyme solution showed that this value did not alter with time. The percentage hydrolyses of α - and β -methyl *N*-acetylglucosaminides are given in Table IV.

Table IV

Time (hr.)	% splitting	
	β -Methyl <i>N</i> -acetylglucosaminide	α -Methyl <i>N</i> -acetylglucosaminide
3	6	0
6	10	0
24	35	0
48	56	0
65	—	0

It appears, therefore, that α -methyl *N*-acetylglucosaminide is not split by *Helix pomatia* enzyme under the conditions of the above experiment.

Action of Helix pomatia enzyme on methylglucosaminide hydrochloride. Samples containing 5 mg. of β -methylglucosaminide hydrochloride, 0.2 ml. of enzyme solution, and 0.2 ml. buffer (pH 4.0) were made up to 2 ml. with distilled water. These were analysed for reducing sugar by Hanes' method at zero time and after 24 hr. No free sugar beyond the initial value was found.

Helix pomatia glucosaminidase therefore does not hydrolyse non-acylated glucosaminides.

Action of Helix pomatia enzyme on O-substituted glucosaminides. *N*-acetyl trimethyl- β -methylglucosaminide [Cutler *et al.* 1937] and *N*-acetyl triacetyl- β -phenylglucosaminide [Helferich & Iloff, 1933] were both tested for enzymic hydrolysis, the former by Hanes' method and the latter by the method of Folin & Ciocalteu. In neither case was any hydrolysis observed.

Action of Helix pomatia enzyme on different N-acyl derivatives. The action of the glucosaminidase on *N*-*p*-toluenesulphonyl- β -phenylglucosaminide was determined under slightly different conditions from the other experiments; owing to the low solubility of the substrate, a higher concentration of enzyme was used. After 24 hr. 0.5% splitting was observed, but this is within the limit of experimental error.

Since this compound was not hydrolysed, it was thought to be of interest to discover whether this result was due to a steric effect owing to the very large size of the *N*-substituent; the action of the enzyme on *N*-propionyl and *N*-butyryl β -phenylglucosaminides was therefore studied. In both cases no measurable hydrolysis occurred.

The action of the enzyme on *N*-formyl β -methylglucosaminide was then determined by Hanes' method, a comparative experiment on the hydrolysis of *N*-acetyl β -methylglucosaminide being done simultaneously. A blank determination on the *N*-formyl compound was first done in order to discover whether the *N*-formyl group was removed under the conditions of Hanes' determination, and if so, whether the sodium formate so formed had a reducing value. No reduction was observed.

Each substrate (0.02 g.) was dissolved in 6 ml. water, and 4 ml. of the enzyme solution and 1 ml. sodium phthalate buffer (pH 4.0) were added; a blank experiment was done on the enzyme solution, and it was found that the initial reducing value of the enzyme does not increase with time. For the calculation of percentage hydrolysis, it has been assumed that the molecular equivalent in terms of thiosulphate is the same for *N*-formyl as for *N*-acetyl glucosamine. The results are given in Table V.

Table V

Time (hr.)	% splitting	
	<i>N</i> -acetyl	<i>N</i> -formyl
3	23.7	20.1
5	36.4	31.0
6	40.2	34.6
7½	45.0	40.2
21	69.8	70.5

Separation of β -glucosidase and glucosaminidase in Helix pomatia extract. Attempts were made to separate the β -glucosidase from glucosaminidase by filtration through a column of bauxite [Zechmeister *et al.* 1938]. The filtration was done as follows: Hungarian bauxite roughly ground was mixed with 30% of its weight of fine sand, and 18 g. of the mixture were placed in a tube 10 cm. long, diameter 2 cm., until the height in the tube was 5 cm.; the powder was kept in the

tube with glass wool. 10 ml. *N*/50 acetate buffer pH 4.7 were added, and sucked through the filter under low pressure. As soon as the level of the buffer had reached the top of the bauxite column, 25 ml. of enzyme solution were added. The first 10 ml. of filtrate were discarded, and the filtered enzyme was then collected. Alterations in enzyme content of the solution were tested as follows: the amount of β -glucosidase present was determined by testing the activity of the solution towards β -phenyl *N*-acetylglucosaminide at pH 4.0 and at 38°. The concentrations of substrate, enzyme and buffer were the same in both instances. In two experiments, considerable separation was effected, as can be seen in Tables VI and VII.

Table VI

Time (hr.)	Crude enzyme splitting by		Filtered enzyme splitting by	
	Glucosidase %	Glucosaminidase %	Glucosidase %	Glucosaminidase %
2	14.5	52	0.5	30
4½	23.3	90	0.9	58

Table VII

Time (hr.)	Crude enzyme splitting by		Filtered enzyme splitting by	
	Glucosidase %	Glucosaminidase %	Glucosidase %	Glucosaminidase %
14	100	90	20	65

Several unsuccessful attempts were made to repeat this separation, using bauxite from an unknown source, and also Hungarian bauxite. It appears that very small unknown changes in the conditions of the filtration either allow both enzymes to come through equally, or cause both to be retained on the absorbent.

Effect of the crude and filtered enzyme on chitin. The crude enzyme was found to hydrolyse chitin slowly, 22% hydrolysis being found after 254 hr. The action of the filtered enzyme could not be determined, since it was too unstable to retain its activity for a time sufficient to test on chitin whose enzymic hydrolysis is very slow.

DISCUSSION

It has been found, in studying the hydrolysis of *N*-acetyl β -phenylglucosaminide by *Helix pomatia* enzyme, that up to about 65% hydrolysis, the reaction is approximately unimolecular. The behaviour of the β -glucosidase contained in emulsin towards several substrates follows a similar course. Later slowing of the reaction can easily be explained by the assumption that slow destruction of the enzyme occurs with increasing temperature; this is also suggested by a decrease in the value of the apparent activation energy of the reaction at temperatures above 25°.

Table VIII

Temperature °C.	% splitting	$\frac{k_t}{k_{t_0}}$	Apparent activation energy (cal.)
0	13.1	1.00	$E_{0 \rightarrow 9} = 11020$
9	23.6	1.92	$E_{9 \rightarrow 17.5} = 11540$
17.5	39.3	3.55	
25	54.7	5.64	$E_{17.5 \rightarrow 25} = 10620$
37.5	61.7	6.83	$E_{25 \rightarrow 37.5} = 2830$
54.5	39.3	3.55	

By extrapolation of the data given in Table III, the temperature optimum appears to be at 37.5°, although it may be slightly lower. In any case, the

optimum is not sharp: changes in temperature between 28 and 38° do not greatly alter the amount of hydrolysis.

It appears from Table VIII that $E_{37.5 \rightarrow 54.5}$ will have a negative value. The apparent energy of activation is reasonably constant between 0 and 25°, but falls off enormously between 25 and 38°. The negative value obtained for E at temperatures higher than 30° indicates that the temperature increment of inactivation of the enzyme is much larger than that of the reaction. Actually, the expression energy of activation has no strict physical meaning for enzyme reactions, but it has been used in this instance as a convenient method of determining the temperature increment of the reaction in the range 0 to 25°, where it remains reasonably constant.

The pH optimum for *Helix pomatia* glucosaminidase is 3.6, which is lower than that of any other carbohydrase, and is also considerably lower than the optimum, at pH 5.2, which Karrer & Hofmann found for the hydrolysis of chitin by chitinase in *Helix pomatia*. This, however, does not necessarily indicate that chitinase and β -glucosaminidase are different enzymes, since several instances are known in which the substrate may exert a marked effect on the pH optimum.

Specificity of the Helix pomatia enzyme

The failure of the enzyme to hydrolyse *N*-acetyl α -methylglucosaminide, together with Zechmeister's¹ observation that the same enzyme will not hydrolyse *N*-acetyl α -phenylglucosaminide, shows definitely that it is active only towards β -compounds. Further, a substrate containing a free amino group is not hydrolysed by the enzyme; the same behaviour was also observed by Helferich *et al.* [1934] in the glucosaminidase of emulsin, which will not attack a free phenylglucosaminide.

In order to enable enzymic hydrolysis to proceed, however, it is not sufficient to acylate glucosaminides in a random manner. It is hardly surprising that the enzyme is unable to hydrolyse a glucosaminide which is acylated with an artificial aromatic group like *N*-*p*-toluenesulphonyl; more remarkable is its inability to hydrolyse glucosaminides acylated with propionyl and butyryl groups, which are closely related to the acetyl group. The fact that *N*-formyl β -methylglucosaminide is hydrolysed by the enzyme, at a rate comparable with that of the corresponding *N*-acetyl compound, is of considerable interest in view of Miles & Pirie's observation [1939] that the *N*-formyl group occurs naturally in bacterial antigens, and it is tempting to correlate these facts, namely, that the only acylated glucosaminides hydrolysed by the enzyme are the *N*-formyl and *N*-acetyl derivatives, and that these acyl groups are the only *N*-acyl substituents so far known to occur in nature.

This specificity towards different *N*-acyl substituents may be explained by a steric hindrance of the hydrocarbon chain in *N*-propionyl and *N*-butyryl glucosaminides. It is nevertheless surprising that lengthening of the chain by one methylene group should not only slow down but completely prevent enzymic hydrolysis.

Blocking of the hydroxyl groups on carbon atoms 3, 4 and 6 by methylation or acetylation also prevents enzymic hydrolysis. This seems to indicate that the enzyme requires at least one free hydroxyl group to enable it to combine with the substrate.

¹ Zechmeister & Toth have obtained identical results [1939] as to the stereochemical specificity of the enzyme.

It is interesting to note that, as in the case of β -glucosaminidase in emulsin, the *Helix pomatia* enzyme hydrolyses β -methylglucosaminide much more slowly than β -phenylglucosaminide, the ratio being 1 : 34.

We have previously shown [Neuberger & Pitt Rivers, 1939] that α - and β -methylglucosaminides possess *cis* and *trans* configurations respectively. The determination of chemical specificity justifies the description of the *Helix pomatia* enzyme as a *trans*-*N*-acyl glucosaminidase with restricted *N*-acyl specificity. The instability of the chitinase activity in the purified enzyme preparations which is in contrast with the behaviour of the glucosaminidase activity seems to indicate that the two enzymes are separate.

Configuration of chitin

If Fischer's argument that the hydrolysis of cellobiose by emulsin or by the β -glycosidase of yeast provides adequate evidence of its β -configuration is applied to the hydrolysis of chitin, it may be concluded that this polysaccharide has a β -configuration, since the experiments described above together with those of Zechmeister & Toth [1939], show that the *Helix pomatia* enzyme is specific towards β -compounds. The implicit assumption underlying this argument is that the same enzyme effects the hydrolysis of the polysaccharide and all its intermediates; from our experiments, there is no evidence for or against the validity of this assumption, but experiments by Zechmeister & Toth [1939] indicate that the hydrolyses of chitodextrin and chitobiose are effected by different enzymes or enzyme systems.

The β -configuration of chitin therefore cannot be regarded as finally established; nevertheless, evidence of a different kind points in the same direction. Meyer & Pankow [1935] have shown, by comparison of various physical properties, that chitin and cellulose appear to have similar structures, and from more convincing evidence of X-ray determinations they have found that chitin has a periodicity of 2 sugar units and therefore has a *trans* configuration, which, in the glucosamine series, has been shown by us to correspond to a β -configuration. Further evidence on this point may be found from consideration of the rotation of glucosamine penta-acetates and the acetates of chitobiose and chitotriose. The $[\alpha]_D$ values of α - and β -glucosamine penta-acetates are respectively $+106^\circ$ and $+11^\circ$ in glacial acetic acid (our observations), and the corresponding molecular rotations are $+41,230$ and $+4280$. Chitobiose octa-acetate has been prepared independently by Bergmann *et al.* [1931] and by Zechmeister & Toth [1931], and chitotriose undeca-acetate by the latter authors [1932]. For chitobiose octa-acetate [Zechmeister & Toth, 1931] $[\alpha]_D$ is $+55.3^\circ$ in glacial acetic acid, which gives a molecular rotation of $42,030$. From the method of preparation of Bergmann *et al.*, it may be assumed that the compound is an α -acetate, and it can be argued, by analogy with cellobiose which gives the α -octa-acetate on acetylation, that the rotation due to the glucosamine residue carrying the α -acetate will be approximately the same as that of α -glucosamine penta-acetate. The residual rotation due to the other half of the molecule involved in the biose linkage will be the difference between the value for the whole molecule and that of α -glucosamine penta-acetate, i.e. $+800$. This low value strongly indicates a β -configuration and therefore a β -linkage.

If the same calculation is applied to chitotriose undeca-acetate, with $[\alpha]_D + 33^\circ$ in glacial acetic acid [Zechmeister & Toth, 1932], the molecular rotation for the whole molecule is $31,800$, and therefore the molecular rotation for each of the two glucosamine residues in which C_1 is involved in the polysaccharide link becomes

$-9400 \div 2 = -4700$. This again suggests a β -configuration for these two linked glucosamine residues.

It might be argued that chitobiose octa-acetate is a β -acetate with an α -biose link, but if this were the case chitobiose undeca-acetate would have two α -linkages and one β -acetate group, which would make the molecular rotation very much higher (probably over 70,000).

For the purpose of this argument, it has been assumed that acetylation and etherification on C_4 do not greatly alter its contribution to the total rotation. The rules of optical superposition cannot be expected to apply quantitatively, since this alteration on C_4 is not negligible. Nevertheless, the calculations made above lend strong support to the theory that chitobiose, chitotriose and chitin have a β -configuration.

SUMMARY

1. An enzyme extract has been prepared from *Helix pomatia* whose reaction characteristics, pH and temperature optima have been determined.
2. From hydrolysis experiments on α - and β -*N*-acetyl methylglucosaminides, it has been shown that the enzyme is specific to β -compounds.
3. Some new acylated glucosaminides have been prepared; the enzyme has been found to hydrolyse only the *N*-formyl and *N*-acetyl compounds.
4. The enzyme is unable to hydrolyse non-acylated glucosaminides and glucosaminides which are completely *O*-substituted.
5. Separation of the β -glucosidase and β -glucosaminidase activities of *Helix pomatia* extract has been effected.
6. Enzymic experiments and other evidence bearing on the structure of chitin are discussed.

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