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THE DEPENDENCE OF LYSOZYME ACTIVITY ON pH AND IONIC STRENGTH

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SUMMARY

1. The activity of lysozyme (EC 3.2.1.17) towards cell suspensions of *Micrococcus lysodeikticus* has been examined as a function of ionic strength from pH 4–10.

2. Initial increase in activity and subsequent inhibition of lytic activity with increase of ionic strength was much more marked at the higher pH values.

3. The optimum pH for lysis varied markedly with the ionic strength and the maximum activity attained at pH 9.2 was greater than the maximum activity attained at pH 6.2 or below. These results are in contrast to the cleavage of the tetramer of *N*-acetyl-D-glucosamine (GlcNAc) from chitin.

4. Inhibition of lysozyme by GlcNAc and $(GlcNAc)_2$ was independent of ionic strength at pH 6.2 and 9.2, whilst inhibition by other *N*-acylglucosamines and anomers of methyl and ethyl *N*-acetylglucosaminides decreased markedly as the ionic strength was increased at both pH 6.2 and 9.2. Inhibition was always more effective in alkaline solution.

5. The results are discussed in terms of the electrostatic interaction between positive charges on lysozyme and negative charges on the cell wall, that are absent from the oligosaccharides from chitin.

INTRODUCTION

Lysozyme (EC 3.2.1.17) from hen's egg-white cleaves the $\beta(1\rightarrow4)$-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetyl-D-glucosamine (GlcNAc) in the polysaccharide that forms the backbone of bacterial cell walls[1,2]. This glycosidic cleavage is considered to be the first step in the lysis of bacterial cells, but what is measured is the reduction in turbidity of a suspension of dried cells of *Micrococcus lysodeikticus* in aqueous buffer solutions. The activity of the enzyme preparation is recorded as the initial rate of clearing the turbid suspension. Although the change in turbidity has not been correlated directly with the number of glycosidic bonds split and the use of an insoluble substrate is somewhat unsatisfactory, the enzyme has

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid.

usually been assayed in this manner for several reasons, some of which will be mentioned later.

Hen's egg-white lysozyme also cleaves the $\beta(1\rightarrow4)$-linked oligosaccharides of *N*-acetylglucosamine $(GlcNAc)_n$, obtained by partial acid hydrolysis of chitin. The activity towards the intact insoluble polymer is very small[3], but oligomers containing up to six residues of GlcNAc have been isolated by RUPLEY[4] and studied as useful substrates of lysozyme. A soluble polymeric substrate glycol chitin has been investigated by HAYASHI *et al.*[5] and the extent of hydrolysis followed by changes in viscosity and reducing power. A tetrasaccharide $(GlcNAc\cdot MurNAc)_2$ isolated from cell wall material after partial digestion with lysozyme, has also been studied recently by CHIPMAN *et al.*[6]. Kinetic interpretations of the hydrolysis rates of small substrates are however complicated by the occurrence of transglycosylation.

Physicochemical measurements have been made on lysozyme in the presence of these small substrates. The structure of the enzyme and several enzyme–saccharide complexes has been determined by X-ray crystallography[7]. From the structure of the enzyme–chitotriose complex and examination of the cleavage pattern of several oligomers of GlcNAc and MurNAc, the probable active centre has been located[7,8]. The carboxyl groups of Asp 52 and Glu 35 residues at the probable active site, lie on opposite sides of a cleft in the enzyme surface and are respectively in essentially hydrophilic and hydrophobic regions of the protein. According to the catalytic mechanism proposed by PHILLIPS *et al.*[8] and by VERNON[9] one of those carboxyl groups in the active conformation of the enzyme is ionised, whilst the other is un-ionised and serves as a proton donor for the glycosidic cleavage; this mechanism is similar to that which operates in the non-enzymatic hydrolysis of a glycoside by acid. When both groups are fully ionised or fully protonated the enzyme should be inactive.

Recently it was reported by NEUBERGER AND WILSON[10] that lysozyme is very active as a lytic agent even at pH values as high as 9.2 when all the carboxyl groups in the cleft would be expected to be fully ionised. It was also found that lysozyme was inhibited much more effectively at pH 9.2 than at pH 6.2 by several methyl and ethyl *N*-acetylglucosaminides and *N*-acylglucosamines. From the results of these experiments and examination of the three-dimensional model of lysozyme it was proposed that the glycosides might bind at pH 9.2 to Site A rather than to Sites B and C and that other proton donors near this binding site, perhaps Lys 97, could replace Glu 35 as the proton donor and thus maintain enzymatic activity at pH 9.2. In an attempt to determine the kind of inhibition shown by these compounds we have now examined further the activity of lysozyme under its usual assay conditions and in the presence of various salts and inhibitors and also investigated the nature and variation of the lytic activity and the cleavage of cell wall material as a function of pH. In the subsequent paper we report how chemical modification of the basic groups of lysozyme alters the lytic activity and the results obtained enable the function of basic groups to be assessed.

MATERIALS AND METHODS

General

Hen's egg-white lysozyme, lot 25B-8670, and *Micrococcus lysodeikticus*, lot 74B-1730, were obtained from Sigma Chemical, London. Stock solutions of lysozyme

were prepared in either sodium/potassium phosphate buffer (pH 6.2) or water. Protein concentrations were measured by the absorbance at 280 nm with a Unicam SP 700 recording spectrophotometer. A molar absorbance coefficient at 280 nm, $\varepsilon_M = 36\,000$ mole$^{-1}\cdot$cm^2, was used throughout. PRAISSMAN AND RUPLEY[11] and CHIPMAN *et al.*[12] reported $\varepsilon_M = 35\,500$ and 35 400, respectively. The molecular weight was assumed to be 14 600 (ref. 8). Salts were AnalaR wherever possible.

Buffers

Buffer solutions were prepared from AnalaR compounds and their pH was adjusted to the required values with either HCl or NaOH. When ampholytes or uncharged free bases were weighed out, the amount of added NaOH or HCl determined the ionic strength (I) of the resulting solution.

The following buffers were used. Sodium acetate–HCl (pH 4–5.5); mixtures of NaH_2PO_4 (0.2 M) and K_2HPO_4 (0.2 M), usually mixed to give pH 6.2; $NH_2OH\cdot HCl$–NaOH (pH 6.2); sodium barbitone–HCl ($I = 0.05$) (pH 7.5–9.0); Tris–HCl (pH 7.5–9.0); glycine–NaOH (pH 8.5–9.5), usually 0.1 M in glycine, 0.02 M in sodium glycinate ($I = 0.02$, pH 9.2); ethanolamine–HCl (pH 9.5–10.5); imidazole–HCl–NaOH (pH 6.2) or free base at pH 9.2.

Inhibitors

The preparation and properties of the 2-acylamido-2-deoxy-D-glucoses and the alkyl 2-acetamido-2-deoxy-D-glucosides were reported previously by NEUBERGER AND WILSON[10].

Enzyme assay

Lytic activity of lysozyme. The procedure was essentially that reported earlier[10], based on the method of PRASAD AND LITWACK[13]. A stock suspension of cells of *M. lysodeikticus* in water (2.34 mg/ml) was diluted to 78 μg/ml in a final volume of 3 ml of solution. Enzyme solution (0.10 ml) was added to give an enzyme concentration in the assay of approx. 1 μg/ml (69 nM). The absorbance was recorded on a Unicam SP 700 spectrophotometer at 25° and the decrease in turbidity of the suspension was followed at 450 nm. Tangents were drawn to the absorbance traces over the first 30 sec of lysis when less than 10% lysis had occurred and the traces were almost linear. For purposes of comparison, activities were recorded relative to the activity measured in 0.05 M phosphate buffer (pH 6.2; $I = 0.067$). With an enzyme concentration of 1 μg/ml a slope of 0.115 absorbance units/min was usually obtained. A specific activity can be expressed in terms of ΔA/min per molar concentration of enzyme, and used when enzyme concentrations were not identical. Specific activities were usually $1.7\cdot 10^6$ absorbance units/min per molar solution of enzyme in 0.05 M phosphate (pH 6.2). Inhibition by salts, *etc.* was studied by the final addition of enzyme to a solution of cells *plus* inhibitor in buffer and compared with control activities obtained without the inhibitor. In this study we used a cell suspension of 78 μg/ml; this concentration gave almost half the maximum possible rate and was almost equal to the "apparent affinity constant" (see ref. 14).

Cell wall material. Cell walls of *M. lysodeikticus* were prepared by the method of SHARON AND JEANLOZ[15] except that the cells were broken by shaking with No. 12

Ballotini beads in a Braun cell homogeniser cooled by a stream of CO_2. The cell wall fraction was washed several times with water and finally lyophilised.

When cell walls were used as substrate they were mixed with buffer and a homogeneous suspension was formed by ultrasonic disintegration for 10 sec. Cell wall suspension of 192 μg/ml and lysozyme at 17 μg/ml gave initial absorbance values and rates of cleavage similar to those obtained in the usual assays with whole cells.

Activity towards the tetrasaccharide from chitin. *N,N'*-Diacetylchitobiose $(GlcNAc)_2$, the triose $(GlcNAc)_3$, and tetraose $(GlcNAc)_4$ were prepared by partial acid hydrolysis of chitin and isolated by the method described by RUPLEY[4]. They were homogeneous on charcoal–Celite (50:50, w/w) chromatography and on ascending chromatography on Whatman No. 1 paper in an isoamyl alcohol–pyridine–water (1:1:0.8, by vol.) system. Hydrolysis of $(GlcNAc)_4$ by lysozyme was followed at concentrations similar to those employed by SHARON[16] for the cell wall tetrasaccharide but slightly less than those used by KRAVCHENKO and co-workers[17,18].

$(GlcNAc)_4$ was incubated at 5 mg/ml or 3 mg/ml (in a few cases), at 37° with 0.5 mg/ml lysozyme in 0.05 M phosphate buffer (pH 6.2) in a total volume of 0.5 ml. At various times, *viz.* 2, 4, 5, 7 and 24 h, 0.1-ml samples were withdrawn and diluted into 0.5 ml water. The reaction was stopped either by freezing the solution or using it immediately. The solutions were desalted by passage through short columns of Dowex 50 (H^+) and Dowex 1 (acetate). The saccharides were collected in approx. 6 ml of water and evaporated to dryness on a rotary evaporator at 37°; the residue was dissolved in 0.5 ml of water and stored at −15° prior to analysis.

Samples of 0.1–0.2 ml were analysed for *N*-acetylhexosamines by the Morgan–Elson reaction in borate buffer using Reissig's modification, *e.g.*, 0.1 ml sample, 0.4 ml water, 0.1 ml borate (0.8 M; pH 9), with a heating time of 35 min at 100°. Ehrlich reagent (3.0 ml) was added, the solution was incubated at 37° for 20 min and the absorbance was read at 585 nm. The colour yield was recorded in μg equivalents of GlcNAc from a standard curve determined with 0–25 μg GlcNAc. The products of hydrolysis were characterised by thin-layer chromatography according to the method of POWNING AND IRZYKIEWICZ[19]. 15-μl Samples containing approx. 15 μg saccharide were chromatographed on Kieselgel G in *n*-propanol–water–ammonia (70:30:1, by vol.) solvent. The *N*-acetyloligosaccharides were detected with chlorine in CCl_4. The chromatogram was used to identify the products and to indicate whether transglycosylation and hydrolysis had occurred.

At pH 5.5, 6.2 and 8.0, appreciable amounts of a flocculent precipitate of a chitin polysaccharide had formed (after 18 h), but none was present at pH 9.0, even after 26 h. At pH 9.0, thin-layer chromatography showed that higher soluble oligomers were not present and that hydrolysis had not occurred at this pH value. A reaction time of 4–8 h was suitable for the formation of appreciable amounts of dimer, trimer and monomer and subsequent assays involved making determinations after 4, 8 and 24 h.

Digestion products of M. lysodeikticus cell walls. The products of reaction of lysozyme and cell walls were examined to determine whether the products at pH 6.2 and 9.2 were identical.

Purified cell walls were washed and dialysed against water and then digested with lysozyme (0.05 mg/ml) at pH 6.2 and 9.2 in 0.05 M ammonium acetate at 37°. After 24 h the digests were dialysed against water (3 l), and the dialysates were

lyophilised. Preliminary fractionation of the digestion products was carried out on a column of Sephadex G-15 and samples were then subjected to high-voltage paper electrophoresis (50 V/cm) in pyridine–acetate buffer at pH 6.5, on Whatman 3MM paper. The peptides were detected with ninhydrin and saccharide-containing material detected as fluorescent spots after treatment with alkali[20]. The disaccharide fraction (GlcNAc · MurNAc) was identified with the aid of the data of MIRELMAN AND SHARON[21] and was isolated by preparative high-voltage electrophoresis.

After reduction with $NaBH_4$ in 0.04 M potassium tetraborate and hydrolysis by 2 M hydrochloric acid for 2 h, the products were subjected to high-voltage electrophoresis in pyridine–acetate buffer at pH 3.5. The products were detected with ninhydrin–pyridine and $AgNO_3$ reagents.

RESULTS

The inhibitory capacities of several *N*-acylglucosaminides reported previously had been examined at pH 6.2 in 0.066 M phosphate + 0.1% NaCl ($I = 0.105$) and at pH 9.2 in 0.05 M glycine–NaOH ($I = 0.01$) buffers at greatly differing values of ionic strength. Therefore the activity of lysozyme was re-investigated as a function of the concentration of various buffer ions and of the ionic strength of the solutions over a wide range of pH values. Lysozyme has a high lytic activity at both low (6.2) and high (9.2) pH values, and there is a very marked dependence of the activity on the ionic strength of the aqueous solution. The general form of the variation of activity with I is shown in Fig. 1.

Activity at pH 6.2

Lysozyme is almost inactive in the absence of salts, attains maximum activity at an ionic strength of 0.07 and is strongly inhibited above $I = 0.08$, such that in 0.2 M phosphate only 20% of the maximum activity is observed. After the initial increase in activity subsequent inhibition with increasing I was observed with all the

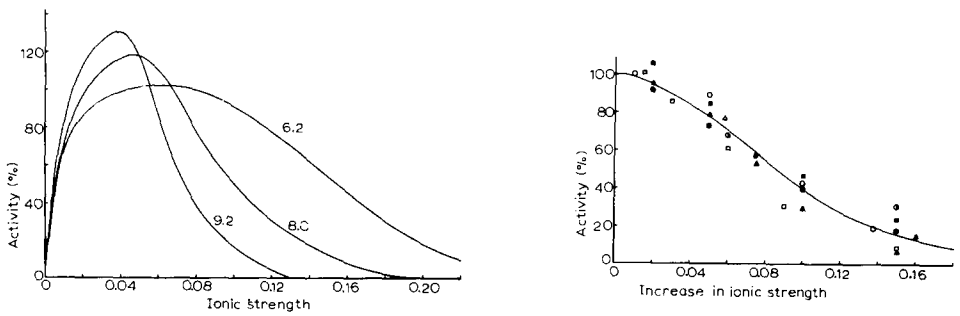

Fig. 1. Lytic activity of hen's egg-white lysozyme as a function of the ionic strength of the solution at pH 6.2, 8.0 and 9.2. Activities are expressed as a percentage of that observed in 0.05 M phosphate (pH 6.2, $I = 0.067$). Concentrations and conditions for the assay are as described in the text.

Fig. 2. Effect of ionic strength of various electrolytes on the activity of lysozyme in 0.05 M phosphate (pH 6.2, $I = 0.067$). $I = \frac{1}{2}\Sigma c_i z_i^2$. ●, NaCl; ○, imidazole hydrochloride + NaCl; ■, $MgSO_4$; □, $MgCl_2$; ▲, KI; △, potassium phosphate; ◑, Na_2SO_4: Activities are expressed as a percentage of that observed in 0.05 M phosphate (pH 6.2, $I = 0.067$).

salts investigated, *viz.* sodium phosphate, potassium phosphate, NaCl, KCl, KBr, KI, Na_2SO_4, imidazole hydrochloride, hydroxylamine hydrochloride, $MgCl_2$ and $MgSO_4$.

For equimolar solutions, bivalent anions (SO_4^{2-}) were almost as effective as inhibitors as bivalent cations (Mg^{2+}), and were much more effective than monovalent anions and cations, yet the combination of bivalent cations and anions produced even greater inhibition. $MgSO_4 \gg MgCl_2 = Na_2SO_4 \gg NaCl$. However in terms of ionic strength, the inhibition was essentially the same with many types of salt, although there were still a few minor variations in effectiveness (Fig. 2). On a basis of ionic strength KI was slightly more inhibitory than KBr, KCl or NaCl, which were slightly better than Na_2SO_4 and not quite as effective as $MgCl_2$. $MgSO_4$ behaved identically with KCl at a quarter of the concentration, yet at the same ionic strength. The points representing 50% inhibition of the activity did not spread over more than 0.04 units at $I = 0.15$.

The dipolar ion of glycine, with an overall charge of zero, did not contribute to the ionic strength and produced no inhibition up to 0.1 M concentration. Imidazole hydrochloride was as effective an inhibitor as NaCl, while neutral imidazole was non-inhibitory.

Activity at pH 9.2

Lysozyme was still a very effective lytic agent in alkaline solutions, even at pH 10. Increasing concentrations of sodium glycinate, chloride, sulphate and potassium phosphate again produced marked reductions in the lytic activity. K_2HPO_4 at pH 9.2 had an effect identical with that of Na_2SO_4. The initial increase and subsequent decrease in lytic activity became very prominent at this pH for bivalent ions, since the ionic strength I is three times the molarity. This inhibition probably accounts for the appearance of a pH optimum of 6.7 in earlier reports of cell lysis in moderately strong phosphate buffers. For 2:1 and 1:1 electrolytes, 50% inhibition was observed at $I = 0.093$ and 0.071 respectively when referred to glycine buffers of $I = 0.02$. The monovalent ions were slightly more effective inhibitors than the bivalent ions, as was found at pH 6.2. However, $MgCl_2$ behaved differently and was a good inhibitor even at 4 mM (pH 9.2). Imidazole at 0.1 M produced no inhibition at all.

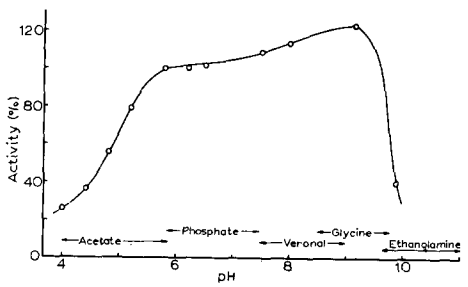

Fig. 3. Lytic activity of lysozyme as a function of pH at ionic strength 0.04–0.05. Activity is recorded as a percentage of the activity at pH 6.2. Buffers (0.01 M) were used with NaCl (0.04 M) at 25° with 78 μg/ml cell suspension and 1 μg/ml enzyme, over the pH ranges indicated.

Activity at other pH values

Similar results were obtained in acetate, veronal, Tris and ethanolamine buffers. In ethanolamine buffer at pH 10 the enzyme was still very active below $I = 0.02$, while in acetate buffer, pH 4, the maximum activity was achieved at $I = 0.1$, although this rate was less than that obtained at pH 6.2.

The observed pH optimum and the ionic strength optimum were intimately related, such that the pH optimum for lysis moved from approximately pH 5 to pH 9, as the ionic strength of the aqueous solution decreased from $I = 0.2$ to $I = 0.02$. At an ionic strength of 0.05 the activity–pH profile remained rather flat from pH 5.8–9.3, whilst the activity was almost at its maximum value (Fig. 3). HAWIGER[22] has recently observed this same phenomenon with a lysozyme from *Staphylococcus aureus*.

Inhibition by neutral sugar derivatives

Inhibition of the lytic activity by the *N*-acylglucosamines and the anomers of ethyl and methyl *N*-acetylglucosaminides are given in Table I for solutions of various ionic strength at pH 6.2 and 9.2. Significant differences were observed at both pH values. The percentage inhibition observed with mutarotated solutions of GlcNAc and $(GlcNAc)_2$ was independent of the ionic strength, whilst the inhibition caused by the other derivatives decreased appreciably as the ionic strength was increased (Fig. 4). Inhibition at pH 9.2 was a little more effective than at pH 6.2. At pH 6.2 the control activities were approximately constant over the range of ionic strength investigated ($I = 0.01$–0.1), whilst at pH 9.2 the control activities were already

TABLE I

EFFECT OF IONIC STRENGTH *(I)* ON THE INHIBITION OF LYSOZYME

Inhibitor	*pH*	*Concn. (mM)*	*Range of I used*	*Variation of inhibition (%)*
N,N'-Diacetylchitobiose	6.2	1	0.02–0.10	32
	9.2	1	0.01–0.06	60
2-Acetamido-2-deoxy-D-glucose	6.2	50	0.02–0.10	58
	9.2	50	0.01–0.08	70
2-Formamido-2-deoxy-D-glucose	6.2	50	0.012–0.10	0
	9.2	50	0.01 and 0.06	0
2-Propionamido-2-deoxy-D-glucose	6.2	40	0.02–0.10	49–1
	9.2	40	0.01–0.08	71–15
Methyl 2-acetamido-2-deoxy-α-D-glucoside	6.2	50	0.02–0.10	54–15
	9.2	50	0.01–0.08	71–19
Methyl 2-acetamido-2-deoxy-β-D-glucoside	6.2	50	0.02–0.10	84–56
	9.2	50	0.01–0.08	97–76
Ethyl 2-acetamido-2-deoxy-α-D-glucoside	6.2	20	0.02–0.10	63–10
	9.2	20	0.01–0.08	65–17
Ethyl 2-acetamido-2-deoxy-β-D-glucoside	6.2	20	0.012–0.10	7–1
	9.2	20	0.01–0.08	62–38

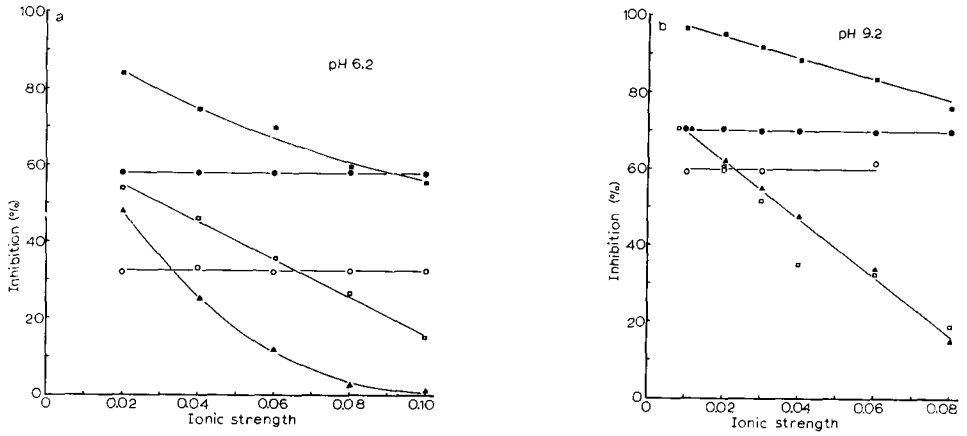

Fig. 4. Inhibition of lysozyme activity by several sugar derivatives with increasing ionic strength at pH 6.2 (a) and pH 9.2 (b). ●, mutarotated GlcNAc; ○, mutarotated *N,N'*-diacetylchitobiose (0.01 M); ■, β-methyl *N*-acetylglucosaminide; □, α-methyl *N*-acetylglucosaminide; ▲, mutarotated *N*-propionyl-D-glucosamine (0.04 M). Concentrations of inhibitor were 0.05 M unless stated. Inhibition is expressed as percentage reduction of control activity determined in the absence of inhibitor at each ionic strength.

significantly inhibited by the salts. The extra inhibition caused by the sugar derivatives however appeared to be similar in effect at both pH values.

As reported earlier, the dimer was the most effective inhibitor studied. β-Methyl *N*-acetylglucosaminide was a good inhibitor, as effective as GlcNAc at $I = 0.1$, and better than the α-anomer at both pH values. Ethyl glycosides, in contrast, were poorer inhibitors than the methyl derivatives and the α-anomer was a more effective inhibitor than the β-anomer at pH 6.2, though not at pH 9.2.

Degradation of cell walls

Purified cell walls were digested at pH 9.2 almost as effectively as at pH 6.2. The variation of activity with salt concentration was less at pH 6.2 and more at pH 9.2 than for the lysis of whole cells. With a suspension of cell walls at 200 μg/ml, 50% inhibition was achieved at $I = 0.35$ and 0.03 at pH 6.2 and 9.2 respectively.

Digestion of cell walls with lysozyme at pH 6.2 and 9.2 in ammonium acetate buffer gave essentially the same products. The patterns obtained by electrophoresis at pH 6.5 of the digests were similar with respect to the peptides and saccharides, except that at pH 9.2 an extra peptide spot was detected. The amount of material that migrated in the position expected of GlcNAc·MurNAc was similar in each digest, and after isolation by preparative paper electrophoresis, the disaccharide from each digest gave glucosamine after reduction and acid hydrolysis. Hence it appears as if the type of enzymatic activity seen at pH 9.2 is the same as that observed in neutral solution.

Cleavage of chitin oligomers

Lysozyme hydrolyses $(GlcNAc)_4$ quite slowly. The rate is 10 times greater than for the cleavage of the trimer but very much less than the rate for the pentamer and hexamer[23,24]. The tetramer was used as a substrate under conditions such that

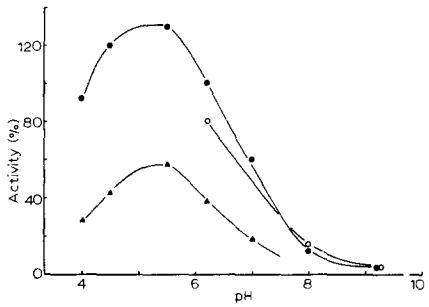

Fig. 5. Hydrolysis of $(GlcNAc)_4$ by lysozyme as a function of pH. 100% activity is equivalent to the release of 10.5 μg equivalents GlcNAc from 100 μg tetrasaccharide after 22 h using the Morgan–Elson colorimetric assay with Reissig's modification. Reaction times, 6.5 h (▲), and 22 h (●) at $I = 0.15$ and $I = 0.05$ (○), 37°. Tetraose concn., 5 mg/ml; lysozyme concn., 0.5 mg/ml.

measurable amounts of $(GlcNAc)_2$ and GlcNAc were liberated over 4–8 h which could be detected by the Morgan–Elson reaction.

At an ionic strength of 0.15 the activity towards the neutral oligomer showed a maximum at pH 5.2 (Fig. 5). At pH 6.5 and pH 8.0 there was only a slight reduction in the activity as the ionic strength was decreased, in marked contrast to the lytic activity. No activity at all was observed at pH 9.2 in solutions of ionic strength at either 0.05 or 0.15. Although the solutions were less concentrated than those used by KRAVCHENKO and co-workers[17,18], the cleavage was again complicated by the occurrence of transglycosylation. Higher soluble oligomers were detected by thin-layer chromatography at pH 6.2 after 2–4 h, but not later, presumably due to the occurrence of further cleavage. At all pH values below 8.5, thin-layer chromatography of the products showed that tetramer, trimer, dimer and small amounts of monomer were present, but at pH 9.2 only the unchanged tetramer was present even after 24 h. Formation of the insoluble chitin-like polymer was observed after 18–24 h at all pH values used except 9.2.

DISCUSSION

The results reported in this paper show that lysis of whole cells and degradation of cell walls of *M. lysodeikticus* by lysozyme occur over a wide pH range, *i.e.*, from pH 4–10, but the rate of lysis is markedly dependent on ionic strength. The ionic strength required for maximum rate of lysis varies with the pH of the solution in such a manner that at the lower pH range the optimum lies at relatively high ionic strength, whilst at the higher pH the optimum is shifted to a relatively low ionic strength. There was an almost complete absence of specific ion effects with the salts investigated. Compounds with zero net charge, such as imidazole and glycine, had no effect on the rate of lysis, although the cationic and anionic forms contributed to the ionic strength and were inhibitory. The observation that lysis of cells occurs not only at slightly acidic or neutral pH, but also at pH 9 had been mentioned by several authors, (see *e.g.*, refs. 23, 25 and 28–31), and confirmed by NEUBERGER AND WILSON[10]. The effects of salts on this lytic reaction had also been noted in several earlier reports[26–31],

but no comprehensive investigation had so far been carried out in which the effects of variations of both pH and ionic strength were systematically tested. It is clear from the results reported here that the pH optimum of 6.7 for lysis, quoted by many investigators, is a result of using particularly strong buffer solutions and that the appearance of two pH optima, reported by NEUBERGER AND WILSON[10], arose from the fact that ionic strength was not kept constant in those earlier experiments.

Kinetic analysis of hydrolysis by lysozyme of GlcNAc oligomers derived from chitin is somewhat difficult, partly because of the occurrence of transglycosylation and also because the available methods for following the cleavage are not very sensitive nor are they based on simple stoicheiometry. However, it could be shown for $(GlcNAc)_4$ that a maximum occurred at pH 5.2 and that there was some enzymatic activity at pH 6.2 and 8.0, but that at pH 9.2 lysozyme was quite inactive on the GlcNAc tetramer. There was a slight influence of ionic strength on the rate of reaction, but it was less marked than with whole cells or cell walls. These results are in agreement with both the pH optimum and the absence of salt effects for the cleavage of the GlcNAc trimer reported by RUPLEY[23,24]. The rate of cleavage of the tetramer in the complete absence of salts was not examined.

In the lysis of cells by lysozyme the first step must be assumed to be the absorption of the lysozyme molecule on to the very large surface of the cell wall. We may also assume that electrostatic forces will play a large part in the binding of the lysozyme. The latter, with an iso-electric point of approximately 11.0, will be strongly positively charged at any pH of the medium, at least up to pH 10.5. We have little quantitative information about ionising groups of the cell wall of *M. lysodeikticus*, but we may assume that it is predominantly negatively charged[32], even at neutral pH, and that this negative charge will increase as the pH is raised. We may thus expect that electrostatic forces will favour absorption of lysozyme by cells, provided the charges of the enzyme and the cell wall are opposite in sign. The favourable electrostatic interaction will be decreased as the ionic strength of the medium is raised and this can explain why higher ionic strength is inhibitory. As the pH is increased the net charge of the lysozyme decreases and to some extent this is probably the reason why, *e.g.* at pH 9.2, an increase of ionic strength from 0.04 to 0.1 leads to a very marked reduction of activity, whilst at pH 6.2 a similar increase in ionic strength is not associated with a significant change in rate of cleavage. The finding that the cleavage of the uncharged tetramer of GlcNAc is not subject to marked inhibition by higher salt concentrations is in accord with this explanation.

Moreover, if the lysozyme molecule is modified chemically in such a way that the positive charges due to lysine and arginine residues are partially or completely masked or removed, enzymatic activity is retained, but activity is only observed at a very low ionic strength and this inhibition becomes even more sensitive to changes in pH (ref. 33). This probably means that the remaining smaller positive charge on the lysozyme results in a decreased attraction to the cell wall and this is more sensitive to competition by the low molecular ions of the buffer.

In the complete absence of salts, or at an ionic strength of 0.005 or less, lysozyme has little or no activity on cell walls, as shown here, and several explanations may be offered.

It has been reported that hydrolysis of glycol chitin[34] and chitin[17] require the presence of small amounts of salts and that interactions of lysozyme with chromato-

graphic columns of neutral chitin[35] and charged CM-chitin[36] are affected by changes in the pH and ionic strength of the media. Thus it is possible that the conformation of the enzyme necessary for activity requires the presence of a certain concentration of small ions, but no direct evidence in favour of this is available at present. In fact all our knowledge of the three-dimensional structure of lysozyme and its complexes is derived from X-ray crystallography of crystals that contain a relatively high concentration of salt[8]. Increasing ionic strength has been reported recently, however, to have slight effects on the instantaneous and 24-h tritium–hydrogen exchange rates of lysozyme[11]. Increasing ionic strength also affects the absorption at 293 nm of the pH difference spectrum of lysozyme assigned to perturbation of the indole chromophores[37], but such effects are not necessarily due to changes in protein conformation.

A more satisfactory explanation for the decreased lytic activity in the absence of salt is that electrostatic binding between lysozyme and cell wall might occur in either a "productive" or an "unproductive" manner, similar to the binding of chitin oligomers[23,24]. Productive binding would thus consist of absorption of lysozyme onto cell wall in such a way that the "backbone" of the cell wall, consisting of alternating residues of GlcNAc and MurNAc, is suitably placed with respect to the catalytically active cleft of the lysozyme molecule for cleavage to take place. Unproductive binding would result if the "glycosidic" part of the cell wall were oriented away from the cleft and on the outside of the lysozyme molecule. That such unproductive binding can indeed occur is indicated by the experiments of FRIEDEN[38] on lysozyme methyl ester. It would then seem possible that the balance between productive and unproductive binding would be largely determined by ionic strength and that the optimum ionic strength for cleavage might vary with the charge on lysozyme and on its distribution over the molecule.

The results obtained on the effect of ionic strength on the inhibitory capacity of GlcNAc, chitobiose, *N*-propionylglucosamine and various *N*-acetylglucosaminides cannot be readily interpreted at the present time and further investigations are clearly needed to elucidate the cause of these differences.

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