INTERACTION OF LYSOZYME WITH ISOMERIC *p*-NITROPHENYL *O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSIDES

A. YA. KHORLIN, E. A. SHASHKOVA, AND S. E. ZURABYAN

M. M. Shemyakin Institute for Chemistry of Natural Products, U. S. S. R. Academy of Sciences, Moscow (U. S. S. R.)

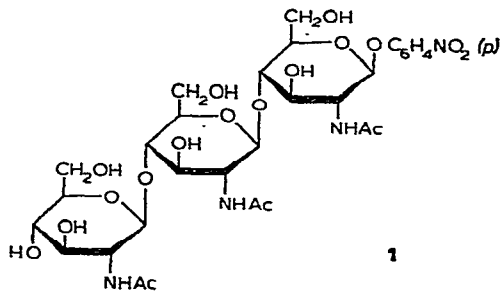
(Received May 6th, 1971; accepted for publication October 1st, 1971)

ABSTRACT

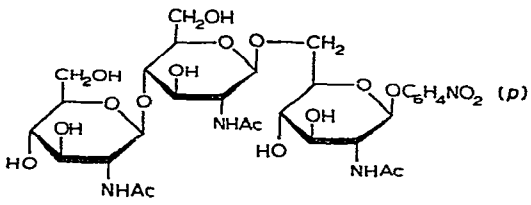
The action of hen egg-white lysozyme on three isomeric trisaccharides, *p*-nitrophenyl tri-*N*-acetylchitotrioside (**1**), *p*-nitrophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranoside (**2**), and *p*-nitrophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranoside (**3**), was investigated. These three compounds exhibited the same inhibitory effect on the lysozyme-catalyzed hydrolysis of *Micrococcus lysodeikticus* cell walls. In addition, **2** and **3** were slowly hydrolyzed by the enzyme with a marked lag period. This suggests that the lysozyme specificity, closely associated with the conformation of the enzyme active site, can vary extensively.

INTRODUCTION

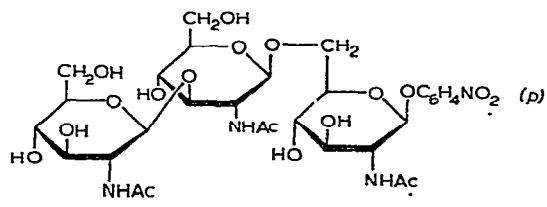
In the kinetic studies of hen egg-white lysozyme (EC 3.2.1.17), polysaccharides (peptidoglycan of *Micrococcus lysodeikticus* cell walls, chitin or its derivatives) and oligosaccharides or their glycosides, that contain β-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl or alternating β-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl and β-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucopyranosyl repeating units are widely used as substrates or competitive inhibitors (for reviews, see Refs. 1 and 2). There is limited information[3–5] about the action of lysozyme on substrates containing another type of glycosidic linkages between 2-acetamido-2-deoxy-D-glucose residues. In order to obtain further information on the specificity requirements of the enzyme, we investigated the action of lysozyme on three isomeric trisaccharides, *p*-nitrophenyl tri-*N*-acetyl-β-chitotrioside (**1**), *p*-nitrophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranoside (**2**), and *p*-nitrophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-



1



2



3

(1→6)-2-acetamido-2-deoxy-β-D-glucopyranoside (**3**). Compounds **1–3** exhibited the same affinity to the active site of lysozyme and all three were hydrolyzed slowly. These results suggest that there is no strict specificity of lysozyme for the (1→4)-position of 2-acetamido-2-deoxy-β-D-glucopyranoside bonds between monosaccharide units in substrates.

EXPERIMENTAL

Hen egg-white lysozyme was prepared and purified as reported by Fevold and Alderton[6]. Compound **1**, m.p. 238–241° (dec.), $[\alpha]_D^{25}$ −24° (*c* 0.4, water), was synthesized after the method of Osawa and Nakazawa[7]. Compound **2**, m.p. 239–241° (dec.), $[\alpha]_D^{25}$ −47° (*c* 1.0, water), and compound **3**, m.p. 209–210° (dec.), $[\alpha]_D^{25}$ +8° (*c* 0.2, water), were synthesized according to Zurabyan *et al.*[8,9]. Di-*N*-acetylchitobiose (**4**) and tetra-*N*-acetylchitotetraose (**5**) were isolated by gel chromatography[10]. Bacterial cell walls of *M. lysodeikticus* were prepared and purified according to Litwack and Pramer[11].

The affinity of compounds **1–3** for the active site of the enzyme was measured by determination of their inhibitory action on the lysis of *M. lysodeikticus* cell walls.

The enzymic hydrolysis was performed in 66mM phosphate buffer, pH 6.20, in the presence of toluene. Other reaction conditions are given in the legends to the Figures. The rate of cell-wall lysis in the absence and in the presence of compounds **1–3** was determined by the light-scattering method[12]. Enzymic hydrolysis of compounds **2** and **3** in the same buffer solution was followed by spectrophotometric determination at 400 nm[13] of the *p*-nitrophenol released in the reaction mixture.

No liberation of *p*-nitrophenol from **1–3** in buffer solution during 10 days at 37° was observed. The activity of lysozyme, as controlled by the light-scattering method[12] was constant under the incubation conditions.

RESULTS AND DISCUSSION

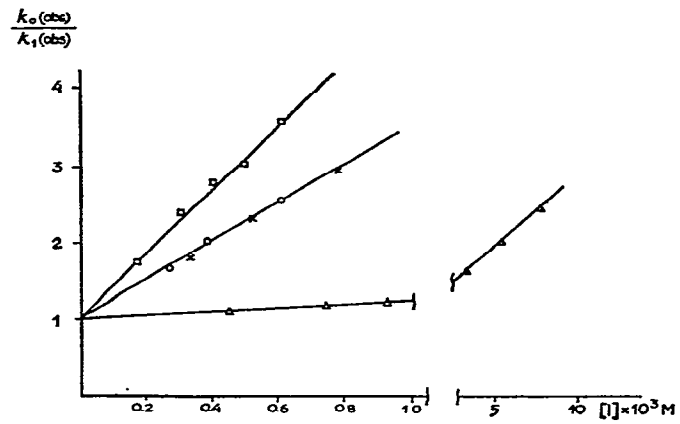


Fig. 1. Inhibition of lysis of *M. lysodeikticus* cell walls (161.5 μg/ml) by lysozyme (3.85 μg/ml) in 66mM phosphate buffer, pH 6.20, at 25°. Compound **1** □, compound **2** ×, compound **3** ○, and compound **4** △. k_0 (obs) and k_1 (obs) are the rate constants in the absence and in the presence of the inhibitors, respectively.

Fig. 1 shows the dependence of lysozyme inhibition on the concentration of compounds **1–4**. The linear character of this dependence indicates that the inhibition is competitive. The $[I]_{50}$ values and K_i values calculated from the ratio $K_i = K_i' \times [I]_{50} \times [I]'_{50}{}^{-1}$ are given in Table I.

Table I shows the similar affinity of **1–3** for the active site of lysozyme. The

TABLE I

INHIBITION BY OLIGOSACCHARIDES OF LYSOZYME ACTION ON *M. lysodeikticus* CELL WALLS

Oligosaccharides	$[I]_{50} \times 10^3$M	$K_i \times 10^5$M
Compound **1**	0.25	0.9
Compound **2**	0.4	1.5
Compound **3**	0.4	1.5
Di-*N*-acetylchitobiose (**4**)	5.0	18.0[a]

[a]The K_i value for **4** is taken for its K_s value[14].

inhibitory activity of the low molecular-weight substrates and inhibitors (oligosaccharides or their glycosides) is most probably determined by formation of nonproductive complexes. In the lysozyme–tri-*N*-acetylchitotriose complex, the monosaccharide units occupy the strong binding subsites A, B, and C of the active site[15]. A similar structure can be assigned to the nonproductive complex lysozyme–compound **1** in which 2-acetamido-2-deoxy-D-glucose residues occupy the subsites A, B and C, and the planar nitrophenyl group is located on the subsite D. Because of the structural and conformational differences between the trisaccharides, an identical binding of these compounds to the active site must be excluded. To explain the strong inhibitory activity of **2** and **3**, it is necessary to assume that attachment of these substances to the active site occurs in a somewhat unusual manner (*cf.* Refs. 16 and 17). It cannot be excluded that this binding can modify the conformation of the active site in enzyme–inhibitor complexes.

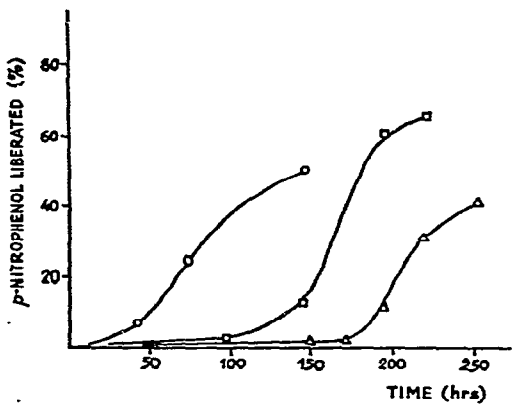


Fig. 2. Kinetics of *p*-nitrophenol liberation in the lysozyme-catalyzed hydrolysis of compounds **2** and **3**. Reaction conditions: 66mM phosphate buffer, pH 6.20, 37°, $[S]_0$ 1.29mM, and $[E]_0$ 5.0 mg/ml. Compound **2** △, compound **3** ○, and compound **2** in the presence of 23% of tetrasaccharide **5** □.

Osawa and Nakazawa[7] have reported the hydrolysis of **1** by lysozyme. The kinetics of *p*-nitrophenol accumulation during the lysozyme-catalyzed hydrolysis of the isomeric trisaccharides **2** and **3** is shown in Fig. 2. It is noteworthy that the kinetics of *p*-nitrophenol liberation during the hydrolysis of **2** and **3** differs from that[7] of **1** by having a marked lag period. This seems to indicate that in the early stages of the hydrolysis of **2** and **3** a direct cleavage gives shorter saccharides, followed by transglycosylation. The resulting higher molecular-weight *p*-nitrophenyl glycosides liberate *p*-nitrophenol at a faster rate than do the starting trisaccharides. This hypothesis is confirmed by the identification of higher oligosaccharides in the incubation mixture of **2** (*cf.* Ref. 12) as well as by a marked decrease of the lag period of *p*-nitrophenol liberation in the enzyme hydrolysis of **2** in the presence of tetra-*N*-acetylchitotetraose.

Thus, it can be concluded that lysozyme specificity, closely associated with the conformation of the enzyme active-site, can vary extensively.

REFERENCES

1 D. M. CHIPMAN AND N. SHARON, *Science*, 165 (1969) 454.
2 M. A. RAFTERY AND F. W. DAHLQUIST, *Fortschr. Chem. Org. Naturst.*, 27 (1969) 340.
3 T. OSAWA, *Carbohyd. Res.*, 1 (1966) 435.
4 J. J. POLLOCK AND N. SHARON, *Biochem. Biophys. Res. Commun.*, 34 (1969) 673.
5 J. J. POLLOCK AND N. SHARON, *Biochemistry*, 9 (1970) 3913.
6 H. L. FEVOLD AND G. ALDERTON, *Biochem. Prep.*, 1 (1949) 67.
7 T. OSAWA AND Y. NAKAZAWA, *Biochim. Biophys. Acta*, 130 (1966) 56.
8 S. E. ZURABYAN, T. P. VOLOSYUK, AND A. YA. KHORLIN, *Carbohyd. Res.*, 9 (1969) 215.
9 S. E. ZURABYAN, T. S. ANTONENKO, AND A. YA. KHORLIN, *Carbohyd. Res.*, 15 (1970) 21.
10 M. A. RAFTERY, T. RAND-MEIR, F. W. DAHLQUIST, S. M. PARSONS, C. L. BORDERS, JR., R. G. WOLCOTT, W. BERANEK, JR., AND L. JAO, *Anal. Biochem.*, 30 (1969) 427.
11 G. LITWACK AND D. PRAMER, *Proc. Soc. Expt. Biol. Med.*, 91 (1956) 290.
12 E. A. SHASHKOVA, I. V. VIKHA, A. A. VICHUTINSKY, AND A. YA. KHORLIN, *Biokhimiya*, 35 (1970) 124.
13 J. W. WOOLLEN, R. HEYWORTH, AND P. G. WALKER, *Biochem. J.*, 78 (1961) 111.
14 F. W. DAHLQUIST, L. JAO, AND M. A. RAFTERY, *Proc. Nat. Acad. Sci. U. S. A.*, 56 (1966) 26.
15 D. M. CHIPMAN, V. GRISARO, AND N. SHARON, *J. Biol. Chem.*, 242 (1967) 4388.
16 C. C. F. BLAKE, L. N. JOHNSON, G. A. MAIR, A. C. T. NORTH, D. C. PHILLIPS, AND V. R. SARMA, *Proc. Roy. Soc., Ser. B*, 167 (1967) 378.
17 C. R. BEDDEL, J. MOULT, AND D. C. PHILLIPS, *Ciba Found. Symp. Mol. Properties Drug Receptors*, (1970) 85.