3,4-Dinitrophenyl Tetra-*N*-acetyl-β-chitotetraoside, a Good Chromophoric Substrate for Hens' Egg-white Lysozyme

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Summary 3,4-Dinitrophenyl tetra-*N*-acetyl-β-chitotetraoside has been synthesised and shown to be a good substrate for lysozyme from hens' egg-white.

ALTHOUGH several chromophoric substrates for hens' egg-white lysozyme have been described[1] they all have disadvantages. Most of them react very slowly and some of them, *e.g.* *p*-nitrophenyl di-*N*-acetyl-β-chitobioside, probably react by a complex pathway involving synthesis of higher oligomers from which the phenol is subsequently released.[1e] A chromophoric substrate which did not have these disadvantages would be valuable for the assay of lysozyme and for investigation of its mechanism of action. To this end we have synthesised 3,4-dinitrophenyl tetra-*N*-acetyl-β-chitotetraoside (I) . The 3,4-dinitrophenyl glycoside was preferred to the 2,4-dinitrophenyl glycoside as the

rate of its spontaneous hydrolysis should be lower. A mixture of the peracetylated α-acetates of NAG-3 through NAG-6 (NAG = *N*-acetylglucosamine) was prepared by acetolysis of chitin[2] and crystallisation. It was separated into its components by chromatography on 210 × 4 cm columns of silicic acid eluting with methanol–chloroform mixtures in a gradient elution from 3 to 15% methanol. In a typical experiment 4·5 g of the crystallised acetolysis mixture yielded 0·2 g NAG-2, 1·09 g NAG-3, 1·50 g NAG-4, 0·58 g NAG-5, and 0·08 g NAG-6 acetates. The acetylated NAG-4 was converted into the impure chloride by treatment with acetyl chloride saturated with dry hydrogen chloride

release of 3,4-dinitrophenol. This obviously makes analysis of the time course of the hydrolysis difficult but the rate of release of 3,4-dinitrophenol at zero time should arise solely from direct hydrolysis as no induction periods are found as sometimes occur in the hydrolysis of *p*-nitrophenyl di-*N*-acetyl-β-chitobioside catalysed by lysozyme.[4] The lysozyme-catalysed hydrolysis of (I) was studied by measuring the initial rate of release of 3,4-dinitrophenol spectrophotometrically at 400 nm. These rates were fitted to the Michaelis–Menten equation with $k_{cat} = 1{\cdot}65 \times 10^{-3}$ s^{-1} and $K_m = 7{\cdot}1 \times 10^{-6}$ mol l^{-1} in citrate buffer with pH 5·08 at 40°. Because these parameters are almost

A comparison of the specificities of some substrates for hens' egg-white lysozyme

Substrate	pH	Temperature	k_{cat}/K_m (l mol^{-1} s^{-1})	Ref.
(I)	5·08	40°	232	This work
NAG-5	5	40°	1200	5
NAG-6	5	40°	11,000	5
2,4-Dinitrophenyl di-*N*-acetyl-β-chitobioside	5·0	35°	0·3	1c
NAG-DG-$C_6H_4NO_2$	5·0	40°	0·0167	1e
p-Nitrophenyl tri-*N*-acetyl-β-chitobioside	5·08	40°	0·20	6
p-Nitrophenyl tetra-*N*-acetylchitotetraoside	5·08	40°	0·95	6
2,4-Dinitrophenyl tri-*N*-acetylchitotrioside	5·08	40°	13	6

to which a small amount of acetic acid had been added to effect solubility and then allowed to react with sodium 3,4-dinitrophenolate[3] in dimethylformamide. Deacetylation of the resulting glycoside was carried out by Zemplen's method and the product purified by chromatography on Sephadex G-15 and crystallised from aqueous methanol. A correct analysis for $C_{38}H_{56}N_6O_{25},4H_2O$ was obtained. The ^1H n.m.r. spectrum of a solution in deuterium oxide showed four signals with chemical shift characteristic of acetamide methyl groups.

Examination of the reaction mixtures from the hydrolysis of (I) in the presence of lysozyme by t.l.c. showed that the cleavage of NAG–NAG bonds occurs at about the same rate as

certainly complex quantities involving *inter alia* the equilibrium constants for non-productive binding, the ratio $k_{cat}/K_m = 232$ l mol^{-1} s^{-1} should be a better measure of the specificity. This value is compared with the corresponding values for NAG-5, NAG-6, and several other chromophoric substrates for lysozyme in the Table. Although (I) had a much higher specificity than other chromophoric substrates, hydrolysis occurs at one fifth and one fiftieth the rate for NAG-5 and NAG-6. (I) is clearly not the ultimate synthetic substrate for lysozyme but it is considerably better than any hitherto reported.

We thank the S.R.C. for a studentship.

(*Received, 12th May* 1972; *Com.* 816.)

[1] (a) T. Osawa, *Carbohydrate Res.*, 1966, **1**, 435; (b) T. Osawa and Y. Nakazawa, *Biochem. Biophys. Acta*, 1966, **130**, 56; (c) G. Lowe; G. Sheppard, M. L. Sinnott, and A. Williams, *Biochem. J.*, 1967, **104**, 893; (d) G. Lowe and G. Sheppard, *Chem. Comm.*, 1968, 529, (e) T. Rand-Meir, F. W. Dalquist, and M. A. Raftery, *Biochemistry*, 1969, **8**, 4206; (f) C. S. Tsai, J. Y. Yang, and S. C. Subbarao *Biochem. J.*, 1969, **114**, 529.

[2] S. A. Barker, A. B. Foster, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, 1958, 2268.

[3] A. F. Hollerman and G. Wilhelmy, *Rec. Trav. chim.*, 1902, **21**, 432; N. V. Sidgwick and W. M. Aldous, *J. Chem. Soc.*, 1921, **119**, 1001.

[4] F. Ballardie, unpublished observations.

[5] Calculated from the results of J. A. Rupley and V. Gates, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **57**, 496.

[6] F. W. Ballardie, unpublished results.