BIOORGANIC CHEMISTRY 6, 483–509 (1977)

Some Studies on Catalysis by Lysozyme[1]

FRANCIS W. BALLARDIE, BRIAN CAPON, MURRAY W. CUTHBERT, AND W. M. DEARIE

Chemistry Department, Glasgow University, Glasgow G12 8QQ, Scotland, United Kingdom

Received April 29, 1977

The preparation of some aryl β-glycosides of β-1, 4-linked oligosaccharides of $(GlcNAc)_n$, $n = 2, 3, 4$, is described. These compounds were tested as substrates for lysozyme from hens' egg white. The best of them, $(GlcNAc)_4$-3,4-DNP, had a value of k_{cat}/K_m which was about one-nintieth that for the hydrolysis of $(GlcNAc)_6$. The pH dependence of k_{cat} and k_{cat}/K_m for the hydrolysis of $(GlcNAc)_4$-3,4-DNP was similar to that for $(GlcNAc)_6$. $(GlcNAc)_4$-3,4-DNP was also a substrate for human lysozyme and lysozyme from ducks' egg white (II and III). An impure sample of $(GlcNAc)_2F$ was prepared and this was hydrolyzed much more rapidly than $(GlcNAc)_2$-2,4-DNP by lysozyme. Compounds of type $(GlcNAc)_{n-1}$(XylNAc)Ar, where $n = 2, 3, 4$, were prepared and found not to be substrates for lysozyme. In the presence of $(GlcNAc)_4$ or $(GlcNAc)_5$, lysozyme-induced hydrolyses of (GlcNAc)-3,4-DNP and (Glc)-3,4-DNP were observed but not of (XylNAc)-3,4-DNP, (6-deoxy-GlcNAc)-3,4-DNP, (6-*F*-GlcNAc)-3,4-DNP, and (6-Cl-GlcNAc)-3,4-DNP. The significance of these results is discussed.

SYNTHETIC SUBSTRATES FOR LYSOZYME

The natural substrate for lysozyme is the cell wall of Gram-positive bacteria which, because of the large number of possible sites of attack, is not an easy substrate to use in physical–organic chemical studies. For this reason a number of workers have used oligosaccharides consisting either of alternating β-1,4-linked MurNac[2] and GlcNAc residues (*1*) or of β-1,4-linked GlcNAc residues (*2, 3*) which undergo a fewer number of reactions in the presence of lysozyme. These suffer from the disadvantages that it is a fairly laborious process to carry out kinetic measurements with them and that if fission occurs at more than one point it is necessary to dissect the overall rates into the rates for the different processes. The use of aryl glycosides of these and related oligosaccharides possesses the obvious advantages of the ease of the kinetic measurements and the fact that only one process is measured spectrophotometrically, namely, fission of the glycosyl–aryloxy bond. The disadvantages are that the mechanism of hydrolysis may be different from that for the natural substrate and that the aryloxy residue may be bound more weakly and/or differently from the corresponding residue in the natural substrate. Nevertheless, in our present state of understanding of the mechanism of

[1] This work was described in part at the "Sexto Seminario Latinoamericano de Quimica," Santiago, Chile, January 16, 1974, the Congress of the Chemical Society, Glasgow, April 7, 1976, and at the meeting of the Organic Reaction Mechanisms Discussion Group, Exeter, U.K., July 20, 1976. Preliminary communications: F. W. Ballardie and B. Capon, *Chem. Commun.*, 828 (1972); B. Capon and W. M. Dearie, *Chem. Commun.*, 371 (1974).

[2] Abbreviations used: MurNAc, *N*-acetyl-muramic acid; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; 2-deoxy-Glc, 2-deoxy-D-glucose; XylNAc, *N*-acetyl-D-xylosamine; 6-deoxy-GlcNAc, 6-deoxy-*N*-acetyl-D-glucosamine; 6-*F*-GlcNAc, 6-deoxy-6-fluoro-D-glucosamine; 6-Cl-GlcNAc, 6-chloro-6-deoxy-D-glucosamine; PNP, *p*-nitrophenyl; DNP, dinitrophenyl.

ISSN 0045–2068

action of lysozyme more information on the mechanism of hydrolysis of any substrate would be valuable.

Prior to this work investigations on the hydrolysis of four chromophoric substrates or types of chromophoric substrate catalyzed by lysozyme had been studied: aryl β-glycosides of $(GlcNAc)_2$ (*4–6*), the *p*-nitrophenyl-β-glycoside of $(GlcNAc)_3$ (*7*), the *p*-nitrophenyl-β-glycoside of (GlcNAc)(Glc) (*8*), and the *p*-nitrophenyl-β-glycoside (GlcNAc)(2-deoxy-Glc) (*8*). All of these compounds are very poor substrates for lysozyme from hens' egg white (see Table 1) with k_{cat}/K_m for the best of them,

TABLE 1

VALUES OF k_{cat}/K_m FOR THE HYDROLYSES OF SOME CHROMOPHORIC SUBSTRATES BY LYSOZYME FROM HENS' EGG WHITE AT pH 5 TO 5.2 AND 40°C[a]

Substrate	k_{cat}/K_m ($sec^{-1}\ M^{-1}$)
$(GlcNAc)_6$	18 000
$(GlcNAc)_3PNP$	0.02[b]
GlcNAc-(Glc)PNP	4×10^{-4}
GlcNAc-(2-deoxy-Glc)PNP	1.7×10^{-2}

[a] Reference (*9*), Table XL.
[b] Estimated value.

$(GlcNAc)_3PNP$ and (GlcNAc)(2-deoxy-Glc)-PNP, about six powers of ten smaller than k_{cat}/K_m for $(GlcNAc)_6$. It was also shown by Rand-Meir *et al.* (*8*) that the pathway for hydrolysis of $(GlcNAc)_2PNP$ is probably complex and involves transglycosylation with the syntheses of higher oligomers. If this were so and if the fission of the glycosyl-*p*-nitrophenoxy bond, the process measured spectrophotometrically, occurs in these higher oligomers, they should be more reactive. We therefore synthesized $(GlcNAc)_3PNP$, studied previously by Osawa and Nakaazawa (*7*), and $(GlcNAc)_4PNP$. The release of *p*-nitrophenol from these compounds and $(GlcNAc)_2PNP$ is shown in Fig. 1. Under the conditions used with $[E]_0 > [S]_0$ release of *p*-nitrophenol from $(GlcNAc)_2PNP$ shows a long induction period consistent with the view that this occurs via a pathway which involves transglycosylation, but release of *p*-nitrophenol from $(GlcNAc)_3PNP$ and $(GlcNAc)_4PNP$ is much faster and no induction periods were discernible. We have never found any induction periods with either of these substrates, and it seems, initially at least, that fission of the glycosyl-*p*-nitrophenoxy bond occurs directly. The initial rates of release of *p*-nitrophenol from $(GlcNAc)_4PNP$ were shown to follow the Michaelis–Menten equation with $k_{cat} = 4.02 \times 10^{-4}\ sec^{-1}$, $K_m = 4.49 \times 10^{-4}\ M$ and $k_{cat}/K_m = 0.90\ M^{-1}\ sec^{-1}$ at pH 5.08 and 50°C. This value is 53 times greater than the value of k_{cat}/K_m for (GlcNAc)(2-deoxy-Glu)PNP, but still 2×10^4 times smaller than k_{cat}/K_m for the hydrolysis of $(GlcNAc)_6$ (see Table 1). The value of the second-order constant for the hydrolysis of $(GlcNAc)_3PNP$ determined at low concentrations of enzyme and substrate at pH 5.08 and 40°C was $0.18\ M^{-1}\ sec^{-1}$. This should be equal to k_{cat}/K_m but the value is nine times greater than that estimated by Imoto *et al.* (*9*) (see Table 1) from the results of Osawa and Nakazawa (*7*). The reason for this discrepancy is not clear.

In order to get better substrates the next step was obviously to use a better leaving group and we explored further the use of 2,4-dinitrophenoxy. Lowe and his co-workers (*5*) had already shown that $(GlcNAc)_2$-2,4-DNP was a better substrate than

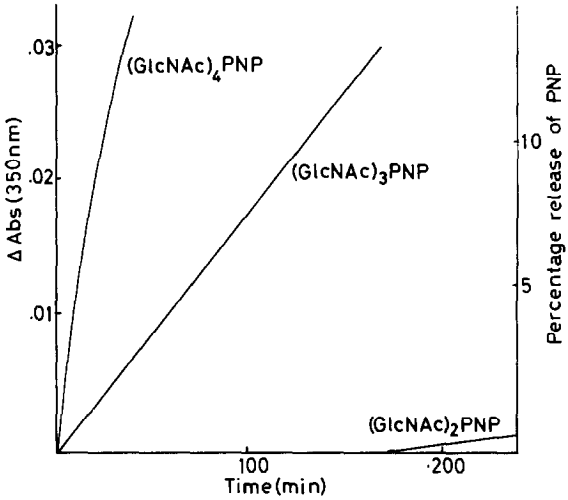


FIG. 1. Release of *p*-nitrophenol from *p*-nitrophenyl glucosides (1.0×10^{-4} *M*) in the presence of lysozyme (2.5×10^{-4} *M*) at 40°C in a citrate buffer, pH 5.08.

$(GlcNAc)_2$PNP, and we prepared this compound and $(GlcNAc)_3$-2,4-DNP. The rates of the lysozyme-catalyzed hydrolyses of both these compounds are considerably greater than those of the corresponding *p*-nitrophenyl glycosides (see Table 2) and neither of them shows an induction period. However, they both have a substantial spontaneous

TABLE 2

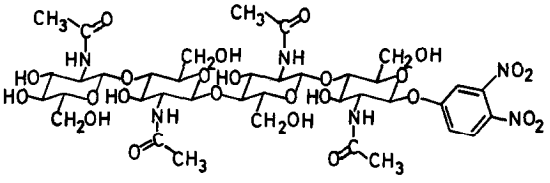
VALUES OF k_{cat}/K_m (M^{-1} sec^{-1}) FOR THE HYDROLYSIS OF SOME ARYL β-GLYCOSIDES OF CHITIN OLIGOSACCHARIDES AT pH 5.08 AND 40°C[a,b]

Aryl group	$(GlcNAc)_2$	$(GlcNAc)_3$	$(GlcNAc)_4$
p-Nitrophenyl	(4×10^{-3})	0.18	0.90
2,4-Dinitrophenyl	0.62[c]	12.5[c]	—
3,4-Dinitrophenyl	0.064	7.9	232

[a] Citrate buffer.
[b] Value for $(GlcNAc)_6$ under similar conditions: 18 000, Refs. (*3*, *9*).
[c] pH 5.1, acetate buffer at 37°C.

rate of hydrolysis, which means that the enzymic rates have to be corrected and that it is not possible to use stock solutions. We therefore sought a leaving group intermediate between *p*-nitrophenoxy and 2,4-dinitrophenoxy and from a consideration of the pK_a's of the dinitrophenols (*10*) it seemed that 3,4-dinitrophenoxy would have the best combination of a high enzymic rate and a low spontaneous rate. The rates of release of

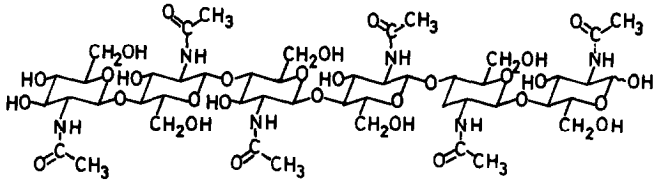
3,4-dinitrophenol from $(GlcNAc)_3$-3,4-DNP and $(GlcNAc)_4$-3,4-DNP (**1**) catalyzed by HEW lysozyme both follow Michaelis–Menten kinetics with k_{cat}/K_m values shown in



$(GlcNAc)_4$3,4DNP

(1)

Table 2. These are about 2300- and 90-fold less than k_{cat}/K_m for $(GlcNAc)_6$ (**2**) under similar conditions.



$(GlcNAc)_6$

(2)

It had originally been hoped to obtain a substrate for lysozyme which was sufficiently reactive for rapid initial burst experiments. However, although $(GlcNAc)_4$-3,4-DNP is the best chromophoric substrate known it is clearly unsuitable for this purpose since it is 90 times less reactive than $(GlcNAc)_6$, which would form the same glycosyl enzyme.

The dependence on pH of k_{cat} and k_{cat}/K_m for the hydrolysis of $(GlcNAc)_4$-3,4-DNP catalyzed by lysozyme from hens' egg white is very similar to that reported for $(GlcNAc)_6$ (see Table 3 and Ref. (*3*)) with a broad maximum at pH 5 to 5.2. This suggests that these substrates are hydrolyzed by the same mechanism.

TABLE 3

VARIATION WITH pH OF k_{cat} AND k_{cat}/K_m FOR THE HYDROLYSIS OF $(GlcNAc)_4$-3,4-DNP BY HENS' EGG WHITE LYSOZYME AT 40°C

pH	$10^3\, k_{cat}$ (sec^{-1})	$10^2\, k_{cat}/K_m$ ($M^{-1}\, sec^{-1}$)
4.02	1.64	0.33
4.40	1.58	2.24
4.88	2.19	2.78
5.03	2.33	3.06
5.04	2.14	1.93
5.08	2.01	2.01
5.19	1.69	3.80
5.35	1.58	2.92
5.71	1.31	2.74
6.08	1.15	1.16
7.03	0.41	0.37

$(GlcNAc)_4$-3,4-DNP is also a substrate for lysozyme from human milk and from duck egg white (II and III). The k_{cat} and K_m values for these enzymes (Table 4) are similar to those for HEW lysozyme except the K_m value for duck egg white lysozyme III, which is appreciably larger than for the other enzymes. K_m is of course a composite constant, but this suggests that $(GlcNAc)_4$-3,4-DNP is bound less strongly to duck egg white III lysozyme than to the other lysozymes. It also appears that $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ are bound less strongly to duck egg III than to duck egg II lysozyme on the basis of investigations of the inhibition of the hydrolysis of bacterial cell wall (*11*).

TABLE 4

COMPARISON OF THE HYDROLYSIS OF $(GlcNAc)_4$-3,4-DNP CATALYZED BY LYSOZYME FROM DIFFERENT SOURCES AT pH 5.08 AND 40°C[a]

	Hen egg white[b]	Human milk[c]	Human milk[d]	Duck egg white II[d]	Duck egg white III[d]
$10^3\, k_{cat}$ (sec^{-1})	2.01	1.35	1.01	1.70	2.26
$10^6\, K_m$ (M)	9.83	7.51	6.80	7.12	32.9
k_{cat}/K_m ($M^{-1}\, sec^{-1}$)	205	180	148	239	60

[a] Acetate buffer, pH 5.08.

[b] Boehringer.

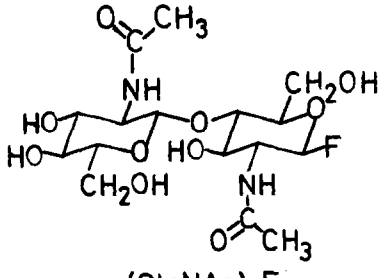
[c] Supplied by Koch Light Laboratories Ltd. from a Japanese source.

[d] Supplied by P. Jollès.

The hydrolysis of $(GlcNAc)_4$-3,4-DNP is not catalyzed by α-lactalbumin.

The observation that k_{cat}/K_m for the hydrolysis of $(GlcNAc)_6$ (**2**) catalyzed by HEW lysozyme is 90 times greater than that for $(GlcNAc)_4$-3,4-DNP (**1**) indicates the importance of binding in sites E and F. The leaving groups for these two substrates are the terminal $(GlcNAc)_2$ residue of the former and the 3,4-dinitrophenoxy group of the latter. Of these, 3,4-dinitrophenoxy would be expected to be the better leaving group and the greater reactivity of $(GlcNAc)_6$ presumably arises from the two terminal GlcNAc residues being bound in sites E and F so that there is a better interaction between the substrate and the catalytic groups of the enzyme.

In order to avoid this type of problem of rotational isomerism about the C–O bonds of the leaving group it was decided to use fluoride, a cylindrically symmetrical leaving group. It has been shown by Barnett *et al.* (*12*) that glycosyl fluorides are frequently substrates for the corresponding glycosidases and (GlcNAc)F has been shown to undergo a rapid neutral hydrolysis which involves neighboring group participation (*13*). A sample of $(GlcNAc)_2F$ (**3**) about 51% pure was prepared and the effect of HEW



$(GlcNAc)_2F$

(3)

lysozyme on its rate of hydrolysis was determined (Table 5). The increased rate of hydrolysis in the presence of lysozyme corresponds to a second-order constant of approximately 20 M^{-1} sec^{-1} at 25°C. Thus, $(GlcNAc)_2F$ is only a slightly poorer substrate for HEW lysozyme than $(GlcNAc)_4$-3,4-DNP, for which the second-order constant is approximately 200 M^{-1} sec^{-1} at 40°C, and much greater than that for $(GlcNAc)_2$-2,4-DNP which is 0.62 M^{-1} sec^{-1} at 37°C. It is not known if this relatively rapid reaction involves neighboring group participation by the amido group as the nonenzymic hydrolysis of (GlcNAc)F (*13*) or whether the pathway is changed in the enzymatically catalyzed reaction.

TABLE 5

EFFECT OF LYSOZYME ON THE RATE OF HYDROLYSES OF $(GlcNAc)_2F$ AT pH 5.0 AND 25.0°C[a]

10^4[Lysozyme] (M)	10^6 [d[acid]/dt][b] (M sec^{-1})	10^6 [d[acid]/dt][c] (M sec^{-1})
0	1.8	—
2	7.5	5.7
4	18.6	16.8
50	Too fast	—

[a] $[(GlcNAc)_2F] = 2 \times 10^{-3}$ M.

[b] Total rate.

[c] Enzymically catalyzed rate after correction for spontaneous rate.

In an attempt to obtain a substrate with a leaving group which would bind in sites E and F, $(GlcNAc)_3$-5-acetamido-2,4-DNP was prepared. The second-order constant for the hydrolysis catalyzed by HEW lysozyme was 4 M^{-1} sec^{-1} at pH 5.08 and 40°C compared to 12.5 M^{-1} sec^{-1} for $(GlcNAc)_3$-2,4-DNP. Clearly just introducing an acetamido group into the 2,4-dinitrophenoxy group of the latter does not lead to more effective binding.

SUBSTRATE DISTORTION AND CATALYSIS BY LYSOZYME

It was proposed by Phillips that in the productive complex of $(GlcNAc)_6$ with lysozyme there is an unfavorable steric interaction between the enzyme and the CH_2OH group of the residue which occupies site D. It was shown that "this overcrowding can be relieved by distortion of the normal chair conformation of residue D toward a conformation with C(6) in an axial position" (*14*). It was thought that this distortion contributed to catalysis since such a conformation should be favorable for an intermediate carbonium ion and the transition state for its formation.

This proposal is related to the earlier suggestion of Haldane (*15*) and Pauling (*16*) that part of the catalytic power of an enzyme derives from its ability to bind the transition state more strongly than the initial state. A thermodynamic analysis of this has recently been published by Fersht (see Fig. 2) (*17*). It was postulated that the standard free energy of activation for the enzymic reaction $\Delta G^{\ddagger}_T$, could be written as the sum of an adverse energy term, $\Delta G^{\ddagger}$, involved in the chemical process of bond breaking

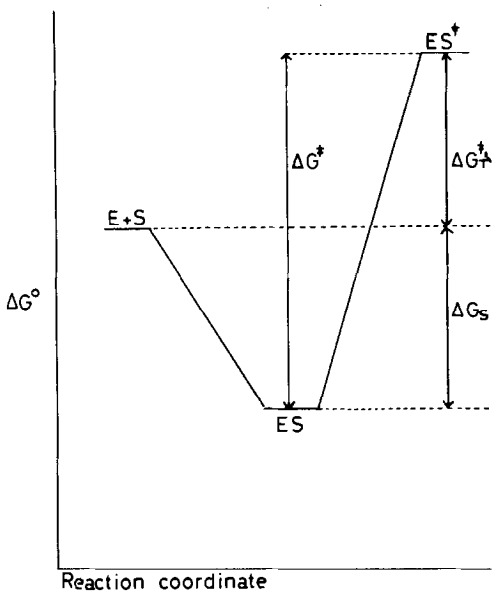


FIG. 2. Change in standard free energy with reaction coordinate for an enzymically catalyzed reaction (cf. Ref. (*17*)).

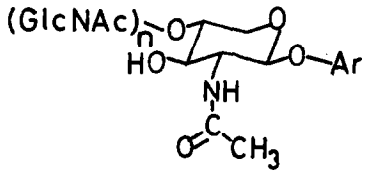
and bond making and an energetically favorable term, ΔG_S, "due to the realization of the enzyme–substrate binding energy,"

$$\Delta G^{\ddagger}_T = \Delta G^{\ddagger} + \Delta G_S.$$

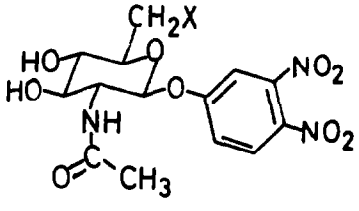
The binding energy of the transition state may be more favorable than that of the initial state for two reasons (or for a combination of these): (1) because there is an energetically favorable interaction between the transition state and the enzyme which does not occur between the initial state and the enzyme; (2) because there is an energetically unfavorable interaction between the initial state and the enzyme which does not occur between the transition state and the enzyme. It is clear, however, that only (1) can lead to a lowering of the standard free energy of activation of the overall reaction, $\Delta G^{\ddagger}_T$, and an increase in k_{cat}/K_m, through a lowering of the free energy level of $ES^{\ddagger}$ (see Fig. 1); (2) would lead to a raising of free energy level of ES and complementary changes in k_{cat} and K_m without affect $\Delta G^{\ddagger}_T$. Therefore a priori substrate distortion in the initial state of the enzyme–substrate complex, which arises from unfavorable steric interactions between substrate and enzyme, cannot affect k_{cat}/K_m. Such distortion would lead to high values of k_{cat} and K_m and to more effective catalysis at high substrate concentrations as described by Fersht; but it cannot lead to a higher value of k_{cat}/K_m. It must be emphasized that this discussion and that of Fersht (*17*) is in terms of rate constants and standard free energies. A complementary discussion to that of Fersht (*17*) in terms of rates and free energies has been given by Jencks (*18*) and as shown by him substrate distortion in the initial-state complex can lead to an increased *rate* of reaction at certain concentrations of substrate.

To provide further information on the structural requirements for catalysis we synthesized a series of substrates similar to those already described but with an *N*-

acetyl-D-xylosamine residue next to the aryloxy group (**4**). These were prepared by the lysozyme-catalyzed transglycosylation of the aryl 2-acetamido-2-deoxy-β-D-xylosides



(4)



(5)

with $(GlcNAc)_4$ or $(GlcNAc)_5$. The products were separated by chromatography on Sephadex and the formation of β-1,4-linkages was confirmed by chemical synthesis of (GlcNAc)(XylNAc)-PNP from (GlcNAc)(XylNAc) prepared by the method of van Eikeren *et al.* (*19*). We could detect no lysozyme-catalyzed hydrolysis with release of phenol with any of these compounds. They all showed a slow spontaneous hydrolysis, and the reproducibility of our measurements of the rate of this enabled us to put an upper limit on the value of k_{cat}/K_m for the lysozyme-catalyzed reaction (see Table 6). The failure to detect hydrolysis was not the result of a rapid cleavage of the (GlcNAc)(XylNAc) bond since some of the original substrate remained even after long reaction times. The difference in the values of k_{cat}/K_m for the hydrolysis of $(GlcNAc)_3$-3,4-DNP and $(GlcNAc)_2$(XylNAc)-3,4-DNP sets a lower limit on the difference in the standard free energy of activation of at least 4.3 kcal mol^{-1}.

TABLE 6

COMPARISON OF THE VALUES OF k_{cat}/K_m (M^{-1} sec^{-1}) AT 40°C AND pH 5.2 OF THE HYDROLYSES OF SOME ARYL GLYCOSIDES CATALYZED BY HENS' EGG WHITE LYSOZYME

$(GlcNAc)_2$(XylNAc) PNP	0	$(GlcNAc)_3$ PNP	0.28[a]
$(GlcNAc)_3$(XylNAc) PNP	0.002[b]	$(GlcNAc)_4$PNP	0.90[a]
(GlcNAc)(XylNAc) PAP	0.004[b]	$(GlcNAc)_2$3,4-DNP	0.064
$(GlcNAc)_2$(XylNAc)3,4 DNP	0.006[b]	$(GlcNAc)_3$3,4-DNP	7.9

[a] At pH 5.08.

[b] Upper limit fixed by reproducibility of measurements of the rate of the spontaneous hydrolysis.

In order to test whether smaller modification of the substrate would cause a smaller reduction in the rate of the enzymically catalyzed reaction we have studied induced hydrolysis of the 3,4-dinitrophenyl glycosides of 2-acetamido-2,6-dideoxy-β-D-glucose

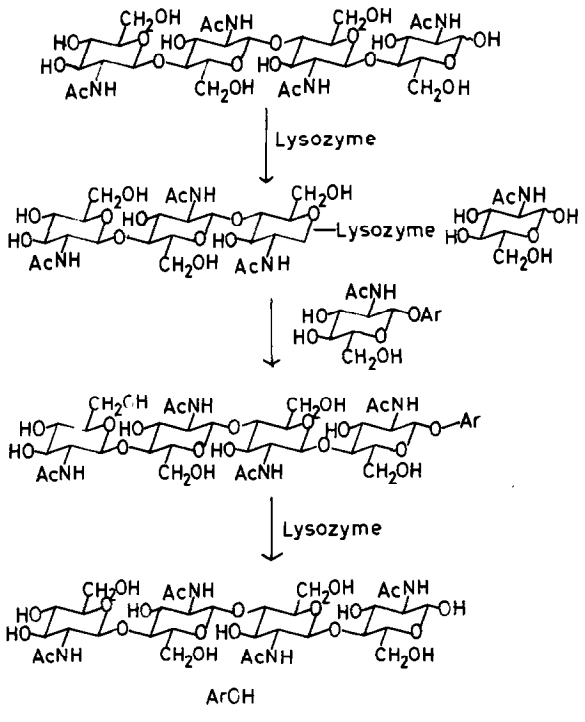
(**5**, X = H), 2-acetamido-6-chloro-2,6-dideoxy-β-D-glucose (**5**, X = Cl), and 2-acetamido-2,6-dideoxy-6-fluoro-β-D-glucose (**5**, X = F) in the presence of lysozyme and $(GlcNAc)_4$ or $(GlcNAc)_5$. The Cl, F, and H substituents (*X*) were chosen as they have larger, approximately equal, and smaller sizes than the OH group as measured by the C–X bond lengths in CH_3–X and the van der Waals radii of X (Table 7). As described in the Experimental section no induced hydrolysis could be detected with these compounds, nor with 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-xyloside, (XylNAc)-3,4-DNP, under condition where rapid release of 3,4-dinitrophenol was observed from 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucoside [(GlcNAc)-3,4-DNP] and 3,4-dinitrophenyl β-D-glucoside (Glc-3,4-DNP). The induced release of 3,4-dinitrophenol from the last two compounds is analogous to the results reported by Raftery and Rand-Meir (*20*) on the induced hydrolysis of the corresponding *p*-nitrophenyl glycosides and failure to observe an induced hydrolysis with the 6-deoxy compound (**5**, X = H) is similar to that reported by Osawa (*21*) with the corresponding *p*-nitrophenyl glycoside, but the use of the 3,4-dinitrophenyl glycoside and of $(GlcNAc)_5$ makes the test more sensitive.

TABLE 7

AN INDICATION OF THE SIZE OF THE GROUP *X* IN COMPOUND (**5**)

X	Bond lengths in CH_3X (Å)	Van der Waals radii (Å)
H	1.09	1.2
O	1.43	1.4
F	1.38	1.35
Cl	1.78	1.8

The pathway for the induced hydrolysis of 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucoside is presumably that shown in Scheme 1, which is similar to that proposed by Raftery and Rand-Meir (*20*). Therefore, the absence of an induced hydrolysis with compound (**5**) could arise because: (1) Lysozyme failed to synthesize oligosaccharides which contain the 3,4-dinitrophenoxy group; (2) lysozyme synthesized oligosaccharides with the 3,4-dinitrophenoxy group, but these did not have β-1,4-linkages; (3) lysozyme synthesized oligosaccharides with the 3,4-dinitrophenoxy group which have β-1,4-linkages, but these are not substrates for lysozyme. Oligosaccharides which contain aryloxy groups were detected in the reaction mixture by thin-layer chromatography, so (1) cannot be correct. It seems unlikely that (2) can be correct as transglycosylation reactions nearly always form β-1,4-linkages, if not completely at least partially (*20–22*), and these have been shown to have been formed with derivatives of 2-acetamido-2-deoxy-β-D-glucose and 2-acetamido-2-deoxy-β-D-xylose. Therefore (3) is the most likely explanation. It also seems unlikely that the failure of these compounds to be substrates can be the result of the inductive effect of the substituent X since H has the opposite polarity from Cl and F and yet none of these compounds are substrates. Also the effect of substituents in this position on the rate of the acid-catalyzed hydrolysis of glycosides is relatively small (*23*). Therefore, it seems that modification of CH_2OH group of this type of sub-



Scheme 1

strate leads to a large decrease in k_{cat}/K_m, which arises from an effect other than the inductive effect. These results also show that distortion of the substrate in the initial state cannot be the only result of the interaction of the CH_2OH group of the enzyme and substrate. It follows from the argument presented earlier that if substrate distortion in the initial state were the only factor which arises from the interaction of the CH_2OH group of the substrate with the enzyme, removal of this group should cause complementary changes in k_{cat} and K_m but not change in the value of k_{cat}/K_m. The observation that there is a reduction in k_{cat}/K_m when the CH_2OH group is replaced by H and probably one when it is replaced by CH_3, CH_2F, and CH_2Cl indicates that there must be an energetically favorable interaction between this group and the enzyme in the transition state. However, because of problems of nonproductive binding with lysozyme which make it difficult to evaluate simple rate constants from k_{cat} and K_m, it is uncertain whether this interaction is also present in the initial-state complex. It is possible that it involves a hydrogen bond between the hydroxyl group of the CH_2OH group of the substrate and the enzyme. This could lead to an induced fit (cf. *18*), a more favorable interaction of the reaction center with the catalytic groups, and/or to catalysis through "increased binding of *nonreacting* groups in the transition state" (*24*). It was proposed by Blake and co-workers (*14*) that there is hydrogen bonding between this CH_2OH group and either CO(57) or Glu-35 in the enzyme–substrate complex.

Studies of the binding of oligosaccharides to lysozyme have been interpreted to indicate that binding of a GlcNAc residue in site *D* is energetically unfavorable as a result of the steric interaction between the CH_2OH group and the enzyme. Originally,

the unfavorable free-energy change on binding a GlcNAc residue in site D was considered to be relatively large, i.e., +3 to +6 kcal mol^{-1} (*25*), but more recently a smaller estimate of +2.3 kcal mol^{-1} has been given (*26, 27*). Therefore, if this latter value is correct "there is little or no ground state strain" due to "contacts between the enzyme and the C(5) hydroxymethyl group on the residue in subsite" (*26*). Further, as pointed out above, such an interaction would only cause complementary changes in k_{cat} and K_m. The most important interaction between this CH_2OH group and the enzyme must be an energetically favorable one in the complex of the enzyme with the transition state.

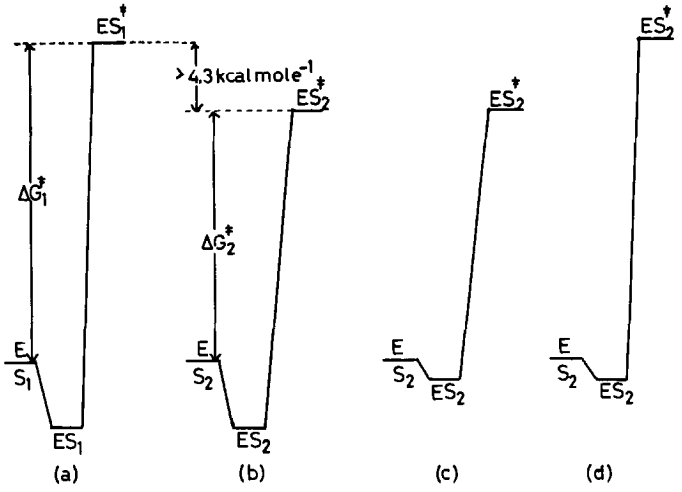


FIG. 3. Possible standard free-energy levels for the interaction of $(GlcNAc)_2(XylNAc)$-3,4-DNP (S_1) and $(GlcNAc)_3$-3,4-DNP (S_2) with lysozyme.

The position is best summarized by the standard free-energy levels given in Fig. 3. Figure 3a is the diagram for $(GlcNAc)_2(XylNAc)$-3,4-DNP (S_1). $\Delta G^{\ddagger}$, the standard free energy of activation, is very high for this reaction reaction as k_{cat}/K_m is very low. If a CH_2OH group is introduced at position 5 of the XylNAc residue we get $(GlcNAc)_3$-3,4-DNP (S_2) and $\Delta G^{\ddagger}$ is reduced by at least 4.3 kcal mol^{-1}. What happens to the energy level of the enzyme–substrate complex is unknown since the rate constant for its conversion into products has not been and probably cannot be determined. Hence the correct diagram for S_2 could be Fig. 3b, where it is unchanged, or Fig. 3c, where it is increased (i.e., the ES_2 complex is strained). However, it is clear that Fig. 3d in which only the level of ES is increased and that of $ES^{\ddagger}$ is unchanged cannot be correct as this leaves $\Delta G^{\ddagger}$ unchanged on going from S_1 to S_2. This argument is independent of the choice of standard state as second-order processes are being compared with one another throughout.

At this stage we do not wish to speculate further on the mechanism of action of lysozyme. In our opinion this is much too ambitious a task in our present state of knowledge. The questions that we should be asking now are "What makes a substrate?" and "What makes an enzyme?" not "What is the mechanism of action?" We are in full agreement with the recent statement of Knowles (*28*) which we paraphrase as "we do not" understand how lysozyme accelerates the rates of the reactions it catalyzes.

EXPERIMENTAL

General Methods

Melting points were determined using a Kofler–Reichert hot-stage melting-point apparatus and are uncorrected.

Nuclear magnetic resonance spectra were measured on a Varian T60 or Varian HA100 spectrometers. Spectra of 220 MHz were obtained from the SRC service. Chemical shifts, unless otherwise stated, are δ values measured relative to internal tetramethylsilane (TMS) and are expressed in parts per million.

Infrared spectra were measured using a Unicam SP 1000, a Unicam SP 200, or a Perkin–Elmer 257 spectrophotometer.

Optical rotations were measured on a Perkin–Elmer 141 polarimeter at 25°C with a cell of 10 cm pathlength using the sodium D line.

Extinction coefficients in the ultraviolet spectrum were determined with a Cary 16 spectrophotometer.

Mass spectra were recorded on an A.E.I MS12 mass spectrometer at 340°C.

Elemental analysis of oligosaccharide derivative with free hydroxyl groups proved difficult as they picked up moisture while being weighed. The figures given below are consistent with there being ca. one molecule of water per sugar residue. Aryl glycosides with a neighboring acetamido group were not dried by heating as this may lead to decomposition.

Chitobiose Octa-acetate

Chitin was acetolysed according to the method of Barker *et al.* (*29*). The product was recrystallized from methanol. Mass spectrum $(M\text{-}59)^+$ at $m/e = 617$ (see Table 8).

TABLE 8

PROPERTIES AND ANALYSES OF PERACETYLATED CHITIN OLIGOSACCHARIDES

			Calculated			Found		
	R_f[a]	mp (lit.[b])	C	H	N	C	H	N
Dimer	0.43	304 (308)	49.7	6.0	4.1	49.7	5.9	4.2
Trimer	0.23	317 (dec.) (305)	49.8	6.0	4.4	49.8	6.1	4.4
Tetramer[c]	0.11	321 (dec.)	49.9	6.0	4.5	50.0	6.0	4.4
Pentamer	0.07	328 (dec.)	50.0	6.0	4.6	49.85	6.0	4.4

[a] tlc on silica, eluting with 10% methanol/chloroform.

[b] Reference (*28*).

[c] $[\alpha]_D = +22.4°C$ (C = 1.0, acetic acid).

Preparation of Peracetylated Chitotriose, Chitotetraose, and Chitopentaose

The acetolysis procedure was a modification of that described by Barker *et al.* (*29*). The exact conditions depended on the quality of chitin used and were optimized to give a maximum yield of peracetylated chitotetraose. Thus, chitin obtained from Eastman Kodak was ball-milled for 2 days, and then dried at 150°C for 1 hr. Analar concentrated sulfuric acid (50 ml) was added slowly to acetic anhydride (500 ml) with

stirring and cooling in a 1-liter round-bottomed flask. The cooled chitin was added to this and left overnight to soak. This mixture was heated at 55°C for 1.25 hr with occasional swirling before working up as described in Ref. (*24*). The crude mixture contained impurities which ran between the oligomeric peracetylated chitin oligosaccharides on tlc (11% methanol/chloroform). These were removed by dissolving the mixture in a minimum of methanol and allowing fractional crystallization of the acetylated oligomers at 0°C for several days. This also served to enrich the mixture in the higher oligomers. These were separated on a 210 × 4.2-cm column of Mallinckrodt Silicic Acid, 100 mesh, activated for 12 hr at 200°C. The column contained 1.8 kg of silicic acid and 4.5 g of the peracetylated oligosaccharide mixture could be separated on this. The column was packed wet, with a thin slurry of the silicic acid in 3% methanol/chloroform. The mixture of acetylated oligosaccharides was applied in a minimum volume of 10% methanol/chloroform and the column was eluted with a progressively increasing polarity of solvent from 3 up to 15% methanol/chloroform, polarity gradient 1.5% methanol/liter. Fractions were collected using an L.K.B. Ultrovac fraction collector over a period of 7–10 days and monitored by tlc (11% methanol/chloroform). Homogeneous fractions were pooled and evaporated, and the peracetylated oligosaccharides were crystallized from methanol (see Table 8). The column would be reused after washing with 2 liters of 3% methanol/chloroform.

The peracetylated oligosaccharides were further characterized through their ir and 220-MHz nmr spectra. The ir spectra all showed the amide I and II bands and a band corresponding to the *O*-acetyl groups. The nmr spectra all showed a signal at δ = ca. 6.2 ppm, J = 2 Hz, showing that they are predominantly α-acetates and signals corresponding to the requisite number of *N*-acetyl groups at δ = ca. 1.93 ppm, *O*-acetyl groups δ = ca. 2.0 to 2.2 ppm, and 1-*O*-acetyl group at δ = ca. 2.24 (α-anomeric acetyl).

De-O-acetylation of the Peracetylated Oligosaccharides

The peracetylated oligosaccharides were deacetylated by adding 0.1 mol of sodium methoxide to a dry methanol/chloroform solution. Partial evaporation of the solvent, followed by cooling, trituration, and the addition of small quantities of ether were often necessary to induce crystallization. Care was also necessary to avoid crystallization of partially de-*O*-acetylated material. The compounds were purified on a 1.5 × 15-cm column of Sephadex G-15, eluting with water. Fractions were monitored by tlc (3:6:2, v/v/v, ammonia, *n*-propanol, water) and those which contained the same oligosaccharides were combined, freeze-dried, and dried in a drying pistol over phosphoric oxide (Table 9).

TABLE 9

PROPERTIES OF CHITIN OLIGOSACCHARIDES

	mp (lit.[a])	$[\alpha]_D$ (lit.[a])
Dimer	205–208 (205–209)	+16.1 (+14.9)
Trimer	305 dec (308–311)	+2.0 (1.8)
Tetramer	285–295 (293–298)	−2.5 (−1.9)
Pentamer	280–290 (296–304)	−7.2 (−6.1)

[a] Reference (*30*).

p-Nitrophenyl Di-N-acetyl-Chitobioside

This was prepared according to the method of Osawa (*4*) and by a lysozyme-catalyzed transglycosylation. For the latter process *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (mp 205–208°C, 0.48 g), tetra-*N*-acetyl-chitotetraose (0.13 g), and lysozyme (0.11 g) were dissolved in citrate buffer (pH 5.1, 50 ml) and dioxane (5 ml). After incubation at 40°C for 20 hr the mixture was chromatographed on Sephadex G-15. *p*-Nitrophenyl di-*N*-acetyl chitobioside (15 mg, mp 220°C, lit. 226–227°C (*4*)) and *p*-nitrophenyl tri-*N*-acetyl chitotrioside (2 mg) were isolated. It was more convenient to prepare the latter compound chemically.

p-Nitrophenyl Tri-N-acetyl Chitotrioside

This material was prepared by the method of Osawa, mp 236–238°C (lit. 238–240°C (*31*)).

p-Nitrophenyl Tetra-N-acetyl Chitotetraoside

Dry, recrystallized chitotetraose peracetate (4.2 g) was suspended in redistilled acetyl chloride (500 ml) and dry ether (100 ml) and saturated with dry HCl at −20°C. Glacial acetic acid (2 ml) was added to help effect solubility. The flask was sealed, clamped, allowed to come to room temperature, and left for 24 hr. The peracetate did not dissolve completely. The flask was cooled to −20°C before opening and the acetyl chloride was removed on a rotatory evaporator at 40°C. The syrupy residue was azeotroped four times with dry benzene (50 ml) to remove the acetic acid. The white powdery residue was run on tlc in 11% methanol/chloroform against starting material. The plate indicated that there had been about 70% conversion to chloride. This crude material was used for the preparation of the glycoside.

Crude peracetyl-chitotetraosyl chloride (1 g) was mixed with sodium *p*-nitrophenolate (0.3 g) and dry dimethylsulfoxide (7 ml) was added. The mixture was shaken at room temperature for 24 hr and poured into ice water. The product was crystallized out, filtered off, washed with sodium bicarbonate solution and distilled water, and recrystallized from methanol/chloroform. Yield of *p*-nitrophenyl peracetyl chitotetraoside 180 mg, 17%, mp 266–267°C, R_f 0.41 (tlc 11% methanol/chloroform), uv λ_{max} 295 nm (methanol).

Anal. Calcd for $C_{56}H_{75}N_5O_{32}$: C, 50.56; H, 5.68; 5.26. Found: C, 50.31; H, 5.42; N, 6.28.

This product was deacetylated by suspending it (70 mg) in dry methanol (1.75 ml) and warming to 40°C. Sodium methoxide solution (0.1 *M*, 0.1 ml) was added and the mixture was shaken. The compound dissolved and reprecipitated in a few minutes. The suspension was left at 0°C overnight, and the deacetylated product was purified by adding distilled water (20 ml) and passing the resulting solution through a 30 × 2-cm column of Sephadex G-15 followed by elution with distilled water. The eluant was monitored by uv, and the homogeneous fractions were pooled, freeze-dried, and dried in a pistol at 40°C. Yield 23 mg = 34%, mp 292–293°C (dec.), λ_{max} 295 nm (water).

Anal. Calcd for $C_{38}H_{57}N_5O_{23} \cdot 5H_2O$: C, 43.80; H, 6.48; N, 6.72. Found: C, 43.22; H, 6.08; N, 6.49.

Release of *p*-nitrophenol after heating in 2 *M* HCl at 100°C for 4 hr = 100.7% of theoretical base on formula of pentahydrate as determined by absorbance at 400 nm after addition of excess NaOH.

Sodium 3,4-Dinitrophenolate

This was prepared by dissolving 3,4-dinitrophenol (*32*) in methanol and adding 95% of the theoretical amount of 1 *M* sodium hydroxide solution. The solvent was removed in a rotatory evaporator and the resulting solid was dried at 40°C/1 mm. *It explodes if heated above this temperature.*

3, 4-Dinitrophenyl Tetra-N-Acetyl-Chitotetraoside

Crude peracetylchitotetraosyl chloride (4.3 g) was mixed with the dried sodium 3,4-dinitrophenolate (3 g) and dimethyl formamide (15 ml) was added. The mixture was shaken for 24 hr at room temperature and then slowly added to 120 ml of ice water. The product was precipitated immediately and filtered off and washed with sodium bicarbonate solution. It was purified by chromatography on silica, eluting with 15% methanol/chloroform, and crystallization from 1:1 methanol/chloroform which contained a small amount of ether. Yield of 3,4-dinitrophenyl peracetyl chitotetraoside 0.735 mg, 18%, mp 241–242°C.

Anal. Calcd for $C_{56}H_{74}N_6O_{34}$: C, 48.91; H, 5.42; N, 6.11. Found: C, 48.18; H, 5.43; N, 5.90.

The product was deacetylated by dissolving in a mixture of dry methanol and chloroform and adding 0.1 *M* sodium methoxide solution. After a few minutes at room temperature the solution was partly evaporated on a rotatory evaporator and water was added. The solution was passed through a column of Sephadex G-15 and the effluent was monitored by uv. The fractions which contained the glycoside were immediately cooled to avoid hydrolysis and mixed with Amberlite MB-1 to remove ionic material. The pooled fractions were filtered and freeze-dried to give a white amorphous powder. Yield 75%, mp 248°C (dec.). This product could with care be recrystallized from methanol–water.

Anal. Calcd for $C_{36}H_{56}N_6O_{25} \cdot 4H_2O$; C, 42.70; H, 6.03; N, 7.86. Found: C, 42.41; H, 5.90; N, 7.20.

uv $\lambda_{max} = 283$ nm, $\varepsilon = 6550$ liter mol^{-1} cm^{-1}. ir showed the absence of *O*-acetyl groups. Amide I and II, 1650 and 1560 cm^{-1}. Aromatic C=C 1610 and 1525 cm^{-1} and broad O–H and N–H stretch 3200–3600 cm^{-1}. The maximum concentration that could be obtained in D_2O was 2×10^{-3} *M*. The nmr spectrum of this solution showed after scanning with the CAT the signals of the amide methyl groups in the ratio 1:2:1 centered at $\delta = 2.66$ ppm.

3,4-Dinitrophenyl Tri-N-Acetyl Chitotrioside

3,4-Dinitrophenyl peracetyl chitotriside was prepared from peracetyl chitotriosyl chloride as for the corresponding tetrameric glycoside, mp 251–252°C.

Anal. Calcd for $C_{44}H_{57}N_5O_{27}$: C, 48.58; H, 5.28; N, 6.44. Found: C, 48.8; H. 5.5; N, 6.0.

ir $\gamma = 3400$, N–H; 1755, C=O; 1676 and 1560 cm^{-1}, amide I and II; and 1610 cm^{-1} aromatic.

De-*O*-acetylation was affected for the corresponding tetrameric glycoside.

Anal. Calcd for $C_{30}H_{43}N_5O_{20} \cdot 3H_2O$: C, 40.23; H, 5.51; N, 7.82; Found: C, 40.07; H, 5.20; N, 7.25.

uv $\lambda_{max} = 283$ nm, $\varepsilon = 6500$ liter mol^{-1} cm^{-1}. The ir spectrum was similar to that of the corresponding tetrameric glycoside.

3,4-Dinitrophenyl Di-N-Acetyl Chitobioside

3,4-Dinitrophenyl peracetyl chitobioside was prepared from peracetyl chitobiosyl chloride (1 g) as for the corresponding tetrameric glycoside. Yield, 0.6 g, 53%, mp 216–217°C.

Anal. Calcd for $C_{32}H_{40}N_4O_{20}$: C, 48.00; H, 5.04; N, 7.00. Found: C, 48.47; H, 5.16; N, 6.81.

De-*O*-acetylation was effected as previously described for the preparation of 2-acetamido-2-2-deoxy-β-D-glucosyl fluoride (*13*). The product was recrystallized from aqueous methanol. $\lambda_{max} = 283$ nm, $\varepsilon = 6560$ liters mol^{-1} cm^{-1}; mp 197–199°C.

Anal. Calcd for $C_{22}H_{30}N_4O_{15} \cdot 3H_2O$; C, 40.99; H, 5.63; N, 8.69. Found: C, 40.93; H, 4.83; N, 8.79.

5-Acetamido-2,4-Dinitrophenyl Tri-N-Acetyl Chitotrioside

Peracetylchitotriosyl chloride (1.1 g) was dissolved in acetone and 2,4-dinitro-5-acetamidophenol (0.48 g) (*33*) and 3.8 ml of sodium hydroxide (0.5 *M*) was added. The mixture was kept at 18°C for 20 hr and evaporated. The syrupy product was purified by preparative tlc eluting with 11% methanol/chloroform, mp 221–222°C.

Anal. Calcd for $C_{46}H_{60}N_6O_{28}$: C, 48.25; H, 5.28; N, 7.34. Found: C, 47.87; H, 5.40; N, 6.95.

This compound was deacetylated as described previously for 2,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucoside (*34*) to yield 5-acetamido-2,4-dinitrophenol tri-*N*-acetyl chitotrioside, ir $\gamma = 3600$–3200, NH and OH; 1660 and 1550 cm^{-1}, amide I and II; uv λ_{max} 276 nm (methanol).

Anal. Calcd for $C_{32}H_{46}N_6O_{21} \cdot 4H_2O$: C, 41.65; H, 5.90; N, 9.11. Found: C, 41.21; H, 5.73; N, 8.87.

2,4-Dinitrophenyl Tri-N-Acetyl Chitotrioside

The per-*O*-acetylated derivative was prepared as described for the corresponding chitobioside (*34*), mp 240–242°C.

Anal. Calcd for $C_{44}H_{57}N_5O_{27}$: C, 48.58; H, 5.28; N, 6.44. Found: C, 48.11; H, 5.26; N, 6.32.

This was deacetylated in a mixture of methanolic hydrogen chloride and chloroform (*34*). uv λ_{max} 275. The ir spectrum showed the absence of *O*-acetates.

Di-N-Acetyl Chitobiosyl-β-Fluoride (**3**)

The peracetyl derivative was prepared from peracetyl chitobiosyl chloride as described previously for tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucosyl fluoride (*13*), mp 210–211°C. Proton nmr ($CDCl_3/CD_3OD$) $\delta = 1.94$–2.20 (21*H* acetyl protons), 3 to 4.2 (15*H*, ring protons), 4.68 (1*H* doublet of doublets $J_{HH} = 5.5$ Hz, J_{HF} 57.0 Hz). Fluorine nmr, doublet of doublets 5610 Hz upfield from external trifluoroacetic acid $J_{H_1F} = 57.0$ Hz, $J_{H_2F} = 11.3$ Hz.

Anal. Calcd for $C_{26}H_{37}FN_2O_{15}$: C, 49.06; H, 5.86, N, 4.40; F, 2.99. Found: C, 49.67; H, 6.00; N, 3.88; F, 2.75.

This compound was de-*O*-acetylated as described for the corresponding monomer (*13*), mp 238–240°C. ir γ 3600–3200 (OH and NH), no signal a 1730–1700 (*O*-acetates), 1660 (amide I), 1565 (amide II). This compound (**3**) was about 51% pure as indicated by its fluorine analysis.

Anal. Calcd for $C_{16}H_{27}FN_2O_{10}$: F, 4.6%. Found: F, 2.28%.

2-Acetamido-1,3,4-Tri-O-acetyl-2-Deoxy-β-D-Xylopyranose

3,4-Di-*O*-acetyl-D-xylal prepared by a standard method (*35*) from 2,3,4-tri-*O*-acetyl-α-D-xylosyl bromide was converted to 3,4-di-*O*-acetyl-2-deoxy-2-nitroso-α-D-xylopyranosyl chloride as described by Lemieux *et al.* (*36*). The action of nitrosyl chloride on a solution of 3,4-di-*O*-acetyl-D-xylal in methylene chloride at −75°C yielded a blue syrup in 90% yield, which was reduced immediately with a zinc–copper couple (*37*) in a solution in acetic anhydride–acetic acid (*36*). The mixture was stirred for 2 days at room temperature, after which it was filtered, the solids were washed with acetic acid–acetic anhydride mixture, and a freshly prepared zinc–copper couple was added to the combined filtrates and stirring was continued. This was repeated every 2 days until the amount of 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-β-D-xylopyranose was at a maximum as shown by tlc (1 : 1, v/v, benzene ethyl acetate). The solution was filtered and the solids were washed with chloroform. The combined filtrates were diluted with chloroform and washed with water, saturated sodium bicarbonate solution, and again with water. The dried chloroform solution was evaporated to give a syrup which was dissolved in methanol and left to stand overnight at 0°C. The first crystals obtained did not have the characteristic amide absorption bands in the ir spectrum and were identified as tetra-*O*-acetyl-2-oximino-D-erythro-pentopyranose, mp 225°C (phase change at 190° to needles), nmr, $\delta = 2.03, 2.06, 2.10$ (12 protons acetyl groups), 5.6–6.2, 4.7–5.4, and 3.3–4.6 ppm (five protons, complicated multiplets). ir (nujol) 1760 (*s*) 1645 (*w*), broad, 1510 (*s*), 1310 (*m*), 1250 (*s*), 1240 (*s*), 1160 (*m*), 1130 (*m*), 1090 (*s*), 910 (*m*), 780 (*w*), 700 (*m*).

Anal. Calcd for $C_{13}H_{17}NO_9$: C, 47.13; H, 5.17; N, 4.23. Found: C, 47.35; H, 5.26; N, 4.43.

On further standing at 0°C the solution yielded further crops of crystals identified as 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-β-D-xylopyranose which was recrystallized from methanol. mp 213–214°C (lit. (*38*) 214–215°C), ir (nujol) $\gamma = 3320$ (*s*), 1750 (*s*), 1740 (*s*), 1665 (*s*), 1525 (*s*), 1315 (*w*), 1300 (*w*), 1220 (*s*), 1110 (*m*), 1075 (*s*), 1040 (*s*), 1010 (*m*), 912 (*m*), 895 (*w*). That this compound had the β-xylo configuration was confirmed by the nmr spectrum (Table 10). The signals were assigned by decoupling experiments at 100 MHz and chemical shifts and coupling constants were determined at 220 MHz. The spectrum is only consistent with a β-xylo configuration and a Cl conformation. A 1C conformation or a lyxo configuration should lead to different coupling constants.

Anal. Calcd for $C_{13}H_{19}NO_8$: C, 49.2; H, 6.0; N, 4.4. Found: C, 49.6: H, 6.25; N, 4.2.

TABLE 10

PROTON MAGNETIC RESONANCE SPECTRUM OF 2-ACETAMIDO-1,3,4-TRI-*O*-ACETYL-2-DEOXY-β-D-XYLOPYRANOSE

Chemical shifts	N-H	H-1	H-2	H-3	H-4	H-5	H-5′
δ values	5.94	5.72	4.25	5.0	4.95	4.15	3.75
Coupling constants	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{4,5'}$	$J_{5,5'}$	
Hz	6	8	8	4	6.25	−12.25	

In a separate experiment 10 g of the tetra-*O*-acetyl-2-oximino-D-erythro-pentopyranose accumulated from several experiments was allowed to react with 40 g of the zinc–copper couple in a mixture of acetic acid (210 ml) and acetic anhydride (30

ml). Fresh zinc–copper couple was added every 2 days as described above. The reaction was followed by tlc and after 8 days the mixture was treated as described above to yield 2-acetamido-1, 3,4-tri-*O*-acetyl-2-deoxy-β-D-xylose (2 g), and unreacted tetra-*O*-acetyl-2-oximino-D-xylo-pentopyranose (3 g).

p-Nitrophenyl 2-Acetamido-3,4-di-O-Acetyl-2-Deoxy-β-D-Xylopyranoside

This compound was prepared either by the method described by Findlay *et al.* (*39*) involving fusion of *p*-nitrophenol with 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-xylose at 120°C with toluene-*p*-sulfonic acid as catalyst, or by the method described by Osawa (*21*) via 2-acetamido-3,4-di-*O*-acetyl-α-D-xylopyranosyl chloride. The latter method gave a slightly better yield (25%) than the former (17%).

2-Acetamido-3,4-di-*O-acetyl*-α-D-xylopyranosyl chloride was prepared by dissolving 2-acetamido-1,3,4-tri-*O*-acetyl-β-D-yxlose (0.9 g) in acetyl chloride (25 ml) and saturating with dry HCl at −10°C. The mixture was left overnight at room temperature in a tightly sealed flask and evaporated to dryness under vacuum. The solid obtained was evaporated three times with dry benzene and then dried under vacuum. A fawn-colored product, mp 120–121°C, was obtained and this was used in the next stage without further characterization or purification. This crude chloride was allowed to react with *p*-nitrophenol as described by Osawa for *p*-nitrophenyl di-*N*-acetyl chitobioside (*21*). The product was recrystallized from ethanol/chloroform to give *p*-nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-xylopyranoside, mp 232°C. The chemical shifts and the apparent coupling constants of the signals from the protons of the sugar ring are given in Table 11. The signals were assigned by decoupling experiments but the δ and J values were taken from the spectrum at 220 MHz. Completely first-order behavior was not observed, but the results suggest that this compound exists as a mixture of conformations with a large proportion in the 1C form.

Anal. Calcd for $C_{17}H_{20}O_9N_2$: C, 51.52; H, 5.09; N, 7.07. Found: C, 52.14; H, 5.24; N, 7.21.

TABLE 11

CHEMICAL SHIFTS AND APPARENT COUPLING CONSTANTS OF THE PROTONS OF THE SUGAR RING OF *p*-NITROPHENYL 2-ACETAMIDO-3,4-DI-*O*-ACETYL-2-DEOXY-β-D-XYLOPYRANOSIDE

Chemical shifts	H-1	H-2	H-3	H-4	H-5	H-5′
δ values	5.43	4.38	5.06	4.93	4.20	3.69
Apparent coupling constants	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{4,5'}$	$J_{5,5'}$
Hz	4	4	6	2	3	−13

p-Nitrophenyl 2-Acetamido-2-Deoxy-β-D-Xylopyranoside

This compound was prepared by the de-*O*-acetylation of the di-*O*-acetate by Zemplen's method (*40*). Recrystallization from methanol gave a product, mp 207–208°C. nmr (pyridine-d_5) δ = 8.20 (*d*, J = 9 Hz, 2*H*), 7.30 (*d*, J = 9 Hz, 2*H*), 5.87 (*d*, J = 6 Hz, 1*H*), 3.8–5 (*m*, 5*H*), 2.07 ppm (*s*, 3*H*).

Anal. Calcd for $C_{13}H_{16}N_2O_7$; C, 50.00; H, 5.16; N, 8.97. Found: C, 49.47; H, 5.01, N, 8.72.

3,4-Dinitrophenyl 2-Acetamido-3,4-di-O-Acetyl-2-Deoxy-β-D-Xylopyranoside

Sodium 3,4-dinitrophenolate (1.3 g) and crude 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-α-D-xylopyranosyl chloride were added to dry dimethylformamide (4 ml). The mixture was shaken overnight at room temperature and then poured into a mixture of ice and water. The precipitate was filtered, washed with water to remove excess sodium 3,4-dinitrophenolate, and dried under vacuum at 40°C. Recrystallization from ethanol–chloroform gave material with mp 175–178°C. nmr ($CDCl_3$–CD_3OD), δ = 8.06 (*d*, *J* = 8.5 Hz, 1*H*), 7.47 (*d*, *J* = 2.5 Hz, 1*H*), 7.34 (*q*, *J* = 8.5, 2.5 Hz, 1*H*), 5.51 (*d*, *J* = 6.5, 1*H*), 5.14 (*m*, $1H_2$), 4.98 (*m*, $1H_4$), 4.33 (*m*, $1H_2$), 4.22 (*m*, $1H_5$), 3.71 (*m*, $1H_5$), 2.16 (*s*, 6*H*), 2.01 (*s*, 3*H*).

Anal. Calcd for $C_{17}H_{19}N_3O_{11}$: C, 46.3; H, 4.3; N, 9.5. Found: C, 46.25: H, 4.53; N, 9.42.

3,4-Dinitrophenyl 2-Acetamido-2-deoxy-β-D-xylopyranoside

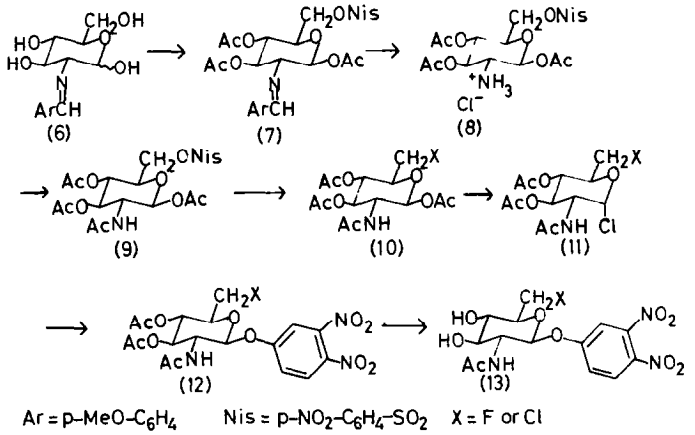
This was prepared from the di-*O*-acetyl compound by Zemplen deacetylation (*40*). The reaction mixture was applied to a column on Sephadex LH20 (30 × 1.5 cm) and eluted with water. Fractions were monitored for the glycoside by ultraviolet absorbance at 284 nm, pooled, and freeze-dried to give a solid, mp 175°C. nmr ([$^2H_5$]pyridine) δ = 8.88 (*d*, *J* = 9 Hz, *NH*), 8.08 (*d*, *J* = 8.5 Hz, *H*), 7.90 (*d*, *J* = 2.5 Hz, 1*H*), 7.50 (*q*, *J* = 8.5, 2.5 Hz, 1*H*), 5.98 (*d*, *J* = 6.5 Hz, 1*H*), 4.62–3.68 (*m*, 5*H*), 2.12 ppm (*s*, 3*H*).

Anal. Calcd for $C_{13}H_{15}N_3O_9$: C, 43.70; H, 4.23; N, 11.76. Found: C, 43.79; H, 4.46; N, 11.58.

Extinction coefficients = 5,500 M^{-1} cm^{-1} at λ = 284 nm.

3,4-Dinitrophenyl 2-Acetamido-2,6-Dideoxy-6-Fluoro-β-D-Glucoside (**13**, *X* = *F*)

This was prepared as shown in Scheme 2, *X* = F. *N-p*-Methoxybenzylidene-2-amino-2-deoxy-D-glucose (**6**) (20 g) (*41*) was dissolved in pyridine and cooled to −10 to −15°C and a solution of *p*-nitrobenzenesulfonyl chloride (17 g) (*42*) in pyridine (60 ml) was added dropwise over a period of 20 min. The reaction mixture was left at 0°C for 18 hr, when acetic anhydride (48 ml) was added dropwise, and and after a further 3 hr



Scheme 2

at room temperature it was poured into an ice–water mixture (3 liter). The precipitate was quickly filtered off, dissolved in chloroform, washed with water, saturated cadmium chloride solution, and water, and dried over magnesium sulfate. Evaporation of the solvent gave a colored oil which crystallized from methanol. Recrystallization from methanol gave 1,3,4-tri-*O*-acetyl-6-*O*-*p*-nitrobenzenesulfonyl-*N*-*p*-methoxybenzylidene-2-amino-2-deoxy-β-D-glucose (**7**). Yield 11 g, mp 155°C.

Anal. Calcd for $C_{25}H_{28}N_2O_{13}S$: C, 50.3; H, 4.7; N, 4.7; S, 5.4. Found: C, 50.1; H, 4.5; N, 4.4; S, 5.5.

The ir and nmr spectra were consistent with the assigned structure.

Hydrochloric acid (4.7 ml, 5 *M*) was added to a refluxing solution of (**7**) (10 g) in acetone (200 ml). Immediately a gelatinous precipitate was formed. After cooling the precipitate was filtered off and washed with a small volume of ice-cold water. Recrystallization from methylated spirits gave 1,3,4-tri-*O*-*acetyl*-6-*O*-*p*-nitrobenzenesulfonyl-2-amino-2-deoxy-β-D-glucose hydrochloride (**8**). Yield 7.1 g, mp 187°C (dec.).

Anal. Calcd for $C_{18}H_{23}ClN_2O_{12}S$: C, 41.0; H, 4.4; N, 5.3; S, 6.1. Found: C, 40.8; H, 4.3; N, 5.3; S, 6.1.

Acetic anhydride (8 ml) was added dropwise to a stirred suspension of (**8**) (5.5 g) in absolute pyridine (24 ml) at 0°C avoiding any sudden rises in temperature. After the reaction mixture had been stirred at room temperature for 4 hr, it was poured into an ice/water mixture (250 ml). A white solid was precipitated, filtered off, washed with water, and dried. Recrystallization from ethanol gave 1,3,4-tri-*O*-acetyl-2-acetamido-2-deoxy-6-*O*-*p*-nitrobenzenesulfonyl-β-D-glucose (**9**). Yield 3.5 g, mp 165°C.

Anal. Calcd for $C_{20}H_{24}N_2O_{13}S$: C, 45.1; H, 5; N, 5.3. Found: C, 45.3; H, 4.8; N, 5.2.

The ir and nmr spectra were consistent with the assigned structure.

A solution of tetrabutyl ammonium fluoride (12 g) (*43*) and (**9**) (4.3 g) in dry acetonitrile (23 ml) was heated at 70°C and the reaction was followed by tlc. After 6 days no starting material could be detected and the solution was concentrated under reduced pressure to yield a black syrupy residue which was chromatographed on a silica column (250 × 50 mm) with ethyl acetate as eluant. 1,3,4-Tri-*O*-acetyl-2-acetamido-2,6-dideoxy-6-fluoro-β-D-glucose (**10**, X = F) was obtained as a white solid which was recrystallized from a mixture of ethanol and diethyl ether, Yield 1.5 g, mp 195–196°C.

Anal. Calcd was $C_{14}H_{20}FNO_8$: C, 48.1; H, 5.8; N, 4.0; F, 5.4. Found: C, 48.2; H, 5.8; N, 4.2; F, 5.4.

The ir and proton nmr spectra were consistent with the assigned structure.

Attempts to prepare this compound from the corresponding 6-*O*-tosylate required much longer reaction times and yielded only products of decomposition.

A solution of (**10**, X = F) (1 g) in freshly distilled acetyl chloride (25 ml) was cooled to −40°C and saturated with dry hydrogen chloride, stoppered and clamped, and left for 18 hr at room temperature. The solution was evaporated under reduced pressure to give a clear syrup which was azeotroped several times with benzene to give crude 3,4-di-*O*-acetyl-2-acetamido-2,6-dideoxy-6-fluoro-α-D-glucosyl chloride (**11**, X = F) which was used immediately in the next stage.

The crude (**11**, X = F) was allowed to react with the sodium salt of 3,4-dinitrophenol (1 g) in *N*,*N*-dimethyl formamide (4 ml) in the same way as described above for the

corresponding 2-acetamido-2-deoxy-xyloside to yield 3,4-dinitrophenyl 3,4-di-*O*-acetyl-2-acetamido-2,6-dideoxy-6-fluoro-β-D-glucoside (**12**, X = F). Yield 0.2 g, mp 200°C.

Anal. Calcd for $C_{18}H_{20}FN_3O_{11}$: C, 45.7; H, 4.3; F, 4.01; N, 8.9. Found: C, 45.4; H, 4.5; F, 4.5; N, 9.0.

The ir and proton nmr spectra were consistent with assigned structure.

A few drops of 1 *M* sodium methoxide were added to a suspension of (**12**, X = F) (0.17 g) in methanol (10 ml). The mixture was left at 0°C for 18 hr and Amberlite IR 120-H resin (1 g) was added. This was filtered off and evaporation of the filtrate gave crude 3,4-dinitrophenyl 2-acetamido-2,6-dideoxy-6-fluoro-β-D-glucoside (**13**, X = F) as a colored solid which was recrystallized from a mixture of methanol and ethanol. Yield 0.084 g, mp 163–165°C (dec.).

Anal. Calcd for $C_{14}H_{16}FN_3O_9 \cdot H_2O$: C, 41.3; H, 4.5; N, 10.3. Found: C, 41.6; H, 4.6; N, 10.3. uv λ 290 nm, ε = 5580 liters mol^{-1} cm^{-1}, ir (KBr disk) 3700–3120 (*s*), 3100 (*m*), 2880 (*m*), 1650 (*s*), 1610 (*s*), 1530 (*s*), 1370 (*s*), 1350 (*s*), 1280 (*s*), 1240 (*s*), 1080 (*s*), 1030 (*s*), 950 (*m*), 890 (*m*), 845 (*m*), 815 (*m*), 750 (*s*) cm^{-1}. nmr (C_5D_5N) δ = 2.16 (*s*, 3*H*); 4.20 (*m*, 2*H*), 4.70 (*m*, 2*H*), 5.06 (broad *s*, 2*H*), 5.10 (*m*, 2*H*, after F decoupling, J_{HF} = 48 Hz), 6.25 (*d*, 1*H*), 7.52–8.18 (*m*, 3*H*), 9.42 (*d*, broad *d*, 1*H*).

3,4-Dinitrophenyl 2-Acetamido-6-Chloro-2,6-Dideoxy-β-D-Glucoside (**13**, X = *Cl*)

This was prepared as shown in Scheme 2, X = Cl. A stirred solution of **9** (1.2 g), anhydrous lithium chloride (0.55 g), hexamethylphosphoramide (4 ml), and sodium-dried toluene (125 ml) was locked under reflux for 6 hr (*44*). The mixture was cooled and filtered, and the filtrate was concentrated under reduced pressure to yield a dark syrupy residue which was chromatographed on a column (210 × 25 mm) of silica, eluting with ethyl acetate. Concentration of the appropriate fractions yielded a white solid which on recrystallization from ethanol yielded 1,3,4-tri-*O*-acetyl-2-acetamido-6-chloro-2,6-dideoxy-β-D-glucose (**10**, X = Cl) (0.25 g) mp 199–200°C (phase change 175°C).

Anal. Calcd for $C_{14}H_{20}ClNO_8$: C, 45.9; H, 5.5; Cl, 9.7; N, 3.8. Found: C, 46.1; H, 5.6; Cl, 9.8; N, 3.2.

The ir and nmr spectra were consistent with the assigned structure.

Compound (**10**, X = Cl) (1 g) was treated with acetyl chloride and hydrogen chloride as described for the preparation of (**13**, X = f) to yield crude 3,4-di-*O*-acetyl-2-acetamido-6-chloro-2,6-dideoxy-α-D-glucosyl chloride (**11**, X = Cl) which was used immediately in the next stage of the reaction.

3,4-Dinitrophenyl 3,4-di-*O*-acetyl-2-acetamido-6-chloro-2,6-dideoxy-β-D-glucoside (**12**, X = Cl) was prepared from (**11**, X = Cl) in a similar manner to (**12**, X = F), mp 170°C.

Anal. Calcd for $C_{18}H_{20}Cln_3O_{11}$: C, 44.1; H, 4.1; Cl, 7.2; N, 8.5. Found: C, 43.9; H, 4.4; Cl, 7.4; N, 8.3.

The ir and nmr spectra were consistent with the assigned structure.

Compound (**12**, X = Cl) (0.32 g) was dissolved in dry methanol (10 ml) and treated with 1 *M* sodium methoxide as described for the preparation of (**13**, X = F) to yield 3,4-dinitrophenyl 2-acetamido-6-chloro-2,6-dideoxy-β-D-glucoside (**13**, X = Cl), yield 0.122 g, mp 166–167°C (dec).

Anal. Calcd for $C_{14}H_{16}ClN_3O_9$: C, 41.4; H, 4.0; N, 10.35. Found: C, 41.2; H, 4.2; N, 10.0.

ir (KBr disk) γ = 3700–3000 (*s*), 1660 (*s*), 1610 (*s*), 1550 (*s*), 1530 (*s*), 1370 (*s*), 1340 (*s*), 1280 (*s*), 1240 (*s*), 1070 (*s*), 845 (*s*), 810 (*s*), 750 (*m*) cm^{-1}.

nmr (C_5D_5N), δ = 2.10 (*s*, 3*H*), 3.96–4.40 (*m*, 4*H*), 4.62 (*m*, 2*H*), 4.75–5.2 (broad *s*, 2*H*), 6.4 (*d*, 1*H*), 7.5–8.0 (*m*, 3*H*), 9.4 (broad *d*, 1*H*).

3,4-Dinitrophenyl 2-Acetamido-2,6-Dideoxy-β-D-glucoside

3,4-Di-*O*-acetyl-2-acetamido-2,6-dideoxy-α-D-glucosyl chloride was prepared by treating 1,3,4-tri-*O*-acetyl-2-acetamido-2,6-dideoxy-β-D-glucose (*41*, *45*) (0.6 g) with acetyl chloride and hydrogen chloride as described previously for the preparation of (**12**, X = F) and allowed to react directly with the sodium salt of 3,4-dinitro-phenol (0.5 g) in dry *N,N*-dimethylformamide (10 ml) to yield 3,4-dinitrophenyl 3,4-di-*O*-acetyl-2-acetamido-2,6-dideoxy-β-D-glucoside, mp 193–194°C.

Anal. Calcd for $C_{18}H_{21}N_3O_{11}$: C, 47.5; H, 4.6; N, 9.2. Found: C, 47.5; H, 4.8; N, 8.95.

This compound was deacetylated with sodium methoxide in methanol as described previously in the preparation of (**13**, X = F), mp 165°C.

Anal. Calcd for $C_{14}H_{17}N_3O_9$: C, 45.3; H, 4.6; N, 11.3. Found: C, 45.2; H, 4.7; N, 11.4.

ir (nujol) γ = 3540–3300 (*s*), 3000 (*s*), 1640 (*s*), 1610 (*s*), 1570 (*s*), 1500 (*m*), 1475 (*s*), 1390 (*s*), 1355 (*s*), 1330 (*m*), 1290 (*s*), 1110 (*s*), 1070 (*s*), 1030 (*s*), 970 (*m*), 890 (*m*), 855 (*m*), 820 (*m*), 765 (*m*), 740 (*m*), 700 (*m*).

nmr (C_5D_5N) δ = 1.56 (*d*, 3*H*), 2.09 (*s*, 3*H*), 3.50–98 (*m*, 1*H*), 4.20–5.14 (*m*, 3*H*), 5.92 (*d*, $J_{1,2}$ = 8 Hz, 1*H*), 7.28–8.04 (*m*, 3*H*), 9.1 (*d*, *J* = 10 Hz, 1*H*).

Formation of Aryl Glycosides of Oligosaccharides by Transglycosylation

Incubation of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside with $(GlcNAc)_4$, $(GlcNAc)_5$, or $(GlcNAc)_6$ in the presence of lysozyme on a small scale was shown by tlc (3:6:2, v/v/v, ammonia, *n*-propanol, water) to yield higher molecular weight compounds which contained the *p*-nitrophenyl group. In a typical experiment *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside (1.5 mg), $(GlcNAc)_5$ (0.62 mg), and lysozyme (0.54 mg) were dissolved in citrate buffer (pH 5.1, 0.25 ml) and dioxane (0.025 ml) at 40°C. Samples were taken every few minutes and applied to a tlc plate. It was estimated that the maximum yield of transglycosylation products was obtained after 30 min. Similar small-scale experiments were performed using $(GlcNAc)_4$ and $(GlcNAc)_6$ when the optimum times for incubation were estimated to be 20 hr and 10 min.

For each of the preparative transglycosylation reactions described below small-scale tlc experiments were performed to estimate the optimum conditions. Separation of the glycosides was achieved by chromatography on a (1.5 × 210 cm) column of Sephadex LH20 eluting with boiled-out distilled water at a flow rate of 12 ml/hr. The effluent was monitored for glycosides using a Cecil Instruments C.E. 212 variable-wavelength ultraviolet monitor set at the wavelength for maximum absorption of the glycoside. The signal from the monitor was fed to a Servoscribe recorder to provide a continuous trace of the absorbance of the effluent. Fractions which contained a glycoside were combined, stirred with Amberlite MB1 resin to remove any traces of reducing sugars, filtered, and rechromatographed on a column (1.5 × 60 cm) of Sephadex G-15. The products were freeze-dried and then dried at room temperature *in vacuo*.

Transglycosylation Reactions with p-Nitrophenyl 2-Acetamido-2-deoxy-β-D-xylopyranoside

p-Nitrophenyl acetamido-2-deoxy-β-D-xylopyranoside (0.15 g), $(GlcNAc)_4$ (0.15 g), and lysozyme (0.024 g) were dissolved in a mixture of citrate buffer (pH 5.1, 30 ml) and spectroscopic grade dioxane (3 ml). After incubation at 40°C for 20 hr the mixture was chromatographed as described above to yield (GlcNAc)(XylNAc)PNP (12 mg), $(GlcNAc)_2$(XylNAc)PNP (4 mg), and $(GlcNAc)_3$(XylNAc)PNP (2 mg).

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside (0.30 g), $(GlcNAc)_5$ (0.15 g), and lysozyme (0.11 g) were dissolved in a mixture of citrate buffer (pH 5.1, 50 ml) and spectroscopic grade dioxane (5 ml). After incubation at 40°C for 30 min the reaction was terminated by the addition of disodium tetraborate buffer (pH 9.7) and the mixture was chromatographed to yield (GlcNAc)(XylNAc)PNP (5 mg), $(GlcNAc)_2$(XylNAc)PNP (6 mg), and $(GlcNAc)_3$(XylNAc)PNP (4 mg).

All the glycosides ran as single spots on tlc (3:6:2, v/v/v, ammonia, *n*-propanol, water).

Characterization of Products of Transglycosylation of p-Nitrophenyl 2-Acetamido-2-deoxy-β-D-xyloside

(GlcNAc)(XyloNAc)-PNP, mp 206–212°C (dec.), nmr (D_2O). Two sharp singlets at $\delta = 2.04$ and 2.08 (*N*-acetyl groups); doublet, $J = 6$ Hz at $\delta = 5.25$ (anomeric proton of XylNAc residue, AA'BB' system at $\delta = 7.2$) and 8.3 (aromatic protons). The rest of the spectrum was obscured by the OH signal. ir (nujol). The spectrum showed characteristic bands at 1635–1660 cm^{-1}, amide I; 1540–1570 cm^{-1}, amide II; and bands due to the aromatic group at 1610, 1595, 1520, and 1488 cm^{-1}. A sample was hydrolyzed with excess sodium hydroxide and the concentration of *p*-nitrophenolate released was calculated from the uv absorbance to be 8.52×10^{-3} *M*. Calculated from weight of material taken 9.7×10^{-3} *M*.

A sample of this compound was also prepared from (GlcNAc)(XylNAc) obtained by the method described by van Eikeren *et al.* (*19*). The crude product (1.1 g) was acetylated and converted to (GlcNAc)(XylNAc)PNP as described by Osawa (*4*) for the preparation of $(GlcNAc)_2$PNP. The product had mp 205–210°C (dec.) which was not depressed in admixture with that of the product obtained enzymically. The two products had identical ir spectra.

$(GlcNAc)_2$(XylNAc)-PNP

Melting point, 255–260°C, nmr (D_2O) three sharp singlets at δ = *ca.* 2 ppm (*N*-acetyl groups), AA'BB' system at $\delta = 7.2$ and 8.3 (aromatic protons). The rest of the spectrum was obscured by the OH signal. The ir spectrum showed characteristic bands of the amide and aromatic groups. A sample was hydrolyzed with excess sodium hydroxide solution. The concentration calculated from the uv spectrum was 4.72×10^{-3} *M*, whereas the concentration of the original by weight was 5.13×10^{-3} *M*.

$(GlcNAc)_3$(XylNAc)-PNP

The ir spectrum showed the characteristic amide and aromatic bands. Hydrolysis in excess sodium hydroxide gave an uv absorption which corresponded to 1.86×10^{-3} *M* when the weighed cencentration was 2.05×10^{-3} *M*.

Transglycosylation Reaction with 3,4-Dinitrophenyl 2-Acetamido-2-deoxy-β-D-xylopyranoside

3,4-Dinitrophenyl 2-acetamido-2-deoxy-β-D-xyloside (0.15 g), $(GlcNAc)_5$ (0.15 g), and lysozyme (0.024 g) were dissolved in citrate buffer (pH 5.1, 30 ml) and spectroscopic grade dioxane (3 ml). After incubation at 40°C for 2 hr the mixture was chromatographed on a column (1.5 × 60 cm) of Sephadex G-15 and the effluent was monitored at 284 nm. This small Sephadex column was used due to the high spontaneous rate of hydrolysis of the 3,4-dinitrophenyl glycosides. However, it did not give complete separation of the transglycosylation products. Three fractions were taken which consisted mainly of trimer, dimer, and monomer glycosides, respectively. These were stirred for 10 min with Amberlite M.B. 1 resin to remove reducing sugars, filtered, and freeze-dried. The fractions when then each dissolved in the minimum amount of water and rechromatographed on a column of Sephadex G-15. This second separation gave the pure glycosides with two and three sugar residues. The compounds separated were pure by tlc (6:3:2, v/v/v, *n*-propanol, ammonia, water). The fastest running compound was identified as the monomer 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-xyloside by comparison with an authentic standard.

(GlcNAc)(XylNAc)-3,4-DNP

The ir spectrum showed the characteristic bands for the amide and aromatic groups. Hydrolysis in sodium hydroxide solution gave a uv absorption which corresponded to a concentration of 1.87×10^{-3} *M* when the weighed concentration was 2.13×10^{-3} *M*.

$(GlcNAc)_2(XylNAc)$-3,4-DNP

The ir spectrum indicated the presence of amide and aromatic groups. Hydrolysis in sodium hydroxide solution gave an uv absorption which corresponded to a concentration of 2.1×10^{-3} *M* when the weighed concentration was 2.4×10^{-3} *M*.

Lysozymes

Lysozyme from hens' egg white was purchased from Boehringer (Batches 7371222 and 6289418). One sample of human lysozyme was supplied by Koch-Light Ltd. from a Japanese source and the other sample and the samples of duck egg white lysozyme II and III were supplied by Professor P. Jollès.

Kinetic Experimental

Kinetic experiments with aryl glycosides were carried out in a Cary 16 spectrophotometer operating on line with Digico Micro 16P minicomputer. Quartz cells with a pathlength of 10 or 2 mm were used in a thermostatted cell block. After initiation of the reaction absorbance value at the λ_{max} of the phenol were gathered on line at a predetermined time interval, and the initial rate of release of phenol was determined by fitting these to a quadratic equation $A_t = a + bt + ct^2$ by the generalized least-squares method (*46*). The initial rate of change of absorbance was converted to the initial rate of reaction by dividing by the extinction coefficient of the released phenol. The computer program was written in a version of Mathchat with a special instruction for servicing the analog–digital converter. The variation of initial rate with substrate concentration was fitted to the Michaelis–Menten equation by the generalized least-squares method (*46*).

The hydrolysis of $(GlcNAc)_2F$ was studied by titrating the released hydrofluoric acid in a pH-stat (*13*).

Induced Hydrolyses

A solution (0.4 ml) which contained (GlcNAc)-3,4-DNP (9×10^{-3} *M*) and $(GlcNAc)_4$ (7.8×10^{-3} *M*) dissolved in a mixture of citrate buffer (pH 5.0, 9 vol) and dioxane (1 vol) was placed in a cell of pathlength 2 mm in a Cary 16 spectrophotometer at 40°C and the rate of spontaneous formation of 3,4-dinitrophenol was measured at 400 nm. A solution of lysozyme (0.1 ml, 2×10^{-3} *M*), was then added and the rate of formation of 3,4-dinitrophenol was measured at various times (Table 12). The maximum rate was reached after 30 to 40 min when it was 7.7 times the rate in absence

TABLE 12

RATE OF FORMATION OF 3,4-DINITROPHENOL FROM A SOLUTION OF (GlyNAc)-3,4-DNP (3.1×10^{-3} *M*), $(GlyNAc)_4$ (6.2×10^{-3} *M*), AND LYSOZYME (4×10^{-4} *M*) IN A MIXTURE OF CITRATE BUFFER (pH 5.0, 0.46 ml) AND DIOXANE (0.04 ml) AT 40°C

Time (min)	10^8 Rate (M sec^{-1})	Percentage reaction[a]
0	1.3[b] (1.6)	0
12	4.0	0.29
25	8.1	0.98
38	10.0	2.3
62	8.8	4.0
88	7.1	5.5

[a] Amount of 3,4-dinitrophenol released expressed as percentage of the starting concentration of (GlcNAc)-3,4-DNP.

[b] Value before addition of lysozyme corrected for dilution; uncorrected value in brackets.

TABLE 13

RATE OF FORMATION OF 3,4-DINITROPHENOL FROM A SOLUTION OF (Glu)-3,4-DNP (2.8×10^{-3} *M*), $(GlcNAc)_4$ (6.77×10^{-3} *M*), AND LYSOZYME (4×10^{-4} *M*) IN A MIXTURE OF CITRATE BUFFER (pH 5.0, 0.46 ml) AND DIOXANE (0.4 ml)

Time (min)	10^9 Rate (M sec^{-1})	Percentage reaction[a]
0	0.	0
21	4.6	0.04
25	7.5	0.08
32	9.6	0.2
37	8.4	0.3

[a] Amount of 3,4-dinitrophenol released expressed as a percentage of the starting concentration of (Glc)-3,4-DNP.

of lysozyme. It is unlikely that the falloff in the rate after this time is due to the decrease in the concentration of the (GlcNAc)-3,4-DNP since this was only 5.5% hydrolyzed after 88 min. It is more likely to be due to a decrease in the concentration of $(GlcNAc)_4$. Similar results were obtained when $(GlcNAc)_5$ was used instead of $(GlcNAc)_4$, except that rate reached a maximum value after 6 min and that the maximum rate of release of 3,4-dinitrophenol was 14 times the spontaneous rate. No induced hydrolyses could be detected with (XylNAc)-3,4-DNP, (6-deoxy GlcNAc)-3,4-DNP, (6-F-GlcNAc)-3,4-DNP, or (6-Cl-GlcNAc)-3,4-DNP in the presence of lysozyme and either $(GlcNAc)_4$ or $(GlcNAc)_5$. An induced hydrolysis was observed with (Glc)-3,4-DNP (see Table 13), however. With this compound no spontaneous hydrolysis could be detected. In the presence of $(GlcNAc)_4$ the maximum rate of the induced hydrolysis was attained after a similar time to that found with (GlcNAc)-3,4-DNP but its value was only about one-tenth that attained with this compound.

ACKNOWLEDGMENTS

We thank the Science Research Council for support, Professor P. Jollès for the gift of human and duck egg white lysozymes, Mr. W. I. Ramage for some preliminary work on the synthesis of compound (**5**, X = Cl), Professor D. Chipman for a copy of Ref. (*26*) prior to publication and Dr J. Thomson for a sample of 3,4-dinitrophenyl β-D-glucoside.

REFERENCES

1. M. R. J. Salton and J. M. Ghuysen, *Biochim. Biophys. Acta* **36**, 552 (1959); N. Sharon, *Proc. R. Soc. Ser., B* **167**, 402 (1967); D. M. Chipman, J. J. Pollock, and N. Sharon, *J. Biol. Chem.*, **243**, 487 (1968); U. Zehavi, J. J. Pollock, V. I. Teichberg, and N. Sharon, *Nature (London)* **219**, 1152 (1968); D. M. Chipman, *Biochemistry* **10**, 1714 (1971).
2. H. P. Lenk, M. Wenzel, and E. Schütte, *Naturwissenschaften* **47**, 516 (1960); M. Wenzel, H. P. Lenk, and E. Schütte, *Z. Physiol. Chem.* **327**, 13 (1962); R. F. Powning and H. Irzykiewicz, *Biochim. Biophys. Acta* **124**, 218 (1966); J. A. Rupley, *Biochim. Biophys. Acta* **83**, 245 (1964); J. A. Rupley, *Proc. Roy. Soc. Ser., B* **167**, 416 (1967); J. A. Rupley and V. Gates, *Proc. Nat. Acad. Sci. USA* **57**, 496 (1967); F. W. Dahlquist and M. A. Raftery, *Nature (London)* **213**, 625 (1967); U. Zehavi and R. W. Jeanloz, *Carbohyd. Res.* **6**, 129 (1968); V. I. Maksimov, V. I. Osipov, and E. D. Kaverzneva, *Biokhimiya* **32**, 1169 (1967).
3. S. K. Banerjee, I. Kregar, V. Turk, and J. A. Rupley, *J. Biol. Chem.* **248**, 4786 (1973); S. K. Banerjee and J. A. Rupley, *J. Biol. Chem.* **250**, 8267 (1975); S. K. Banerjee, E. Holler, G. P. Hess, and J. A. Rupley, *J. Biol. Chem.* **250**, 4355 (1976).
4. T. Osawa, *Carbohyd. Res.* **1**, 435 (1966).
5. G. Lowe, *Proc. Roy. Soc., Ser. B* **167**, 431 (1967); G. Lowe, G. Sheppard, M. L. Sinnott, and A. Williams, *Biochem. J.* **104**, 893 (1967); G. Lowe and G. Sheppard, *Chem. Commun.*, 529 (1968).
6. C. S. Tsai, J. Y. Tang, and S. C. Subbarao, *Biochem. J.* **114**, 529 (1969).
7. T. Osawa and Y. Nakazawa, *Biochim. Biophys. Acta* **130**, 56 (1966).
8. T. Rand-Meir, F. W. Dahlquist, and M. A. Raftery, *Biochemistry* **8**, 4206 (1969).
9. T. Imoto, L. N. Johnson, A. C. T. North, D. C. Phillips, and J. A. Rupley, "The Enzymes" (P. D. Boyer, Ed.), 3rd ed., Vol. VII, p. 665. Academic Press, New York, 1972.
10. G. Kortüm, W. Vogel, and K. Andrussow, "Dissociation Constants of Organic Acids in Aqueous Solutions," pp. 452–453. Butterworths, London, 1961.
11. D. Charlemagne and P. Jollès, *C.R. Acad. Sci., Ser. D* **270**, 2721 (1970).
12. J. E. G. Barnett, W. T. S. Jarvis, and K. A. Munday, *Biochem. J.* **103**, 699 (1967); J. E. G. Barnett, *Biochem. J* **123**, 607 (1976).

13. F. W. BALLARDIE, B. CAPON, W. M. DEARIE, AND R. L. FOSTER, *Carbohyd. Res.* **49**, 79 (1976).
14. 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**, 378 (1967).
15. J. S. B. HALDANE, "Enzymes," p. 182. Longmans, Green and Co., London, 1930.
16. L. PAULING, *Chem. Eng. News* **24**, 1374 (1946); *Amer. Sci.* **36**, 51 (1948).
17. A. R. FERSHT, *Proc. Roy. Soc., Ser. B.* **187**, 397 (1974).
18. W. P. JENCKS, *Advan. Enzymol.* **43**, 219 (1975).
19. P. VAN EIKEREN, W. A. WHITE, AND D. M. CHIPMAN, *J. Org. Chem.* **38**, 1831 (1973).
20. M. A. RAFTERY AND T. RAND-MEIR, *Biochemistry* **7**, 3281 (1968).
21. T. OSAWA, *Carbohyd. Res.* **7**, 217 (1968).
22. J. J. POLLOCK AND N. SHARON, *Biochemistry* **9**, 3913 (1970).
23. See B. CAPON, *Chem. Rev.* **69**, 419 (1969).
24. W. J. RAY AND J. W. LONG, *Biochemistry* **15**, 3993 (1976); G. G. HAMMES, *Nature (London)* **204**, 342 (1964); K. SCHRAY AND J. P. KLINMAN, *Biochem. Biophys. Res. Commun.* **57**, 641 (1964).
25. D. M. CHIPMAN AND N. SHARON, *Science* **165**, 454 (1969); Ref. 9, p. 827.
26. M. SCHINDLER, Y. ASSAF, N. SHARON, AND D. M. CHIPMAN, *Biochemistry* **16**, 423 (1977).
27. See also A. WARSHEL, AND M. LEVITT, *J. Mol. Biol.* **103**, 227 (1976); M. R. PINCUS, S. S. ZIMMERMAN, AND H. A. SCHERAGA, *Proc. Nat. Acad. Sci. USA* **73**, 4261 (1976).
28. J. R. KNOWLES, *FEBS Lett.* **62**, E53 (1976).
29. S. A. BARKER, A. B. FOSTER, M. STACEY, AND J. M. WEBBER, *J. Chem. Soc.*, 2218 (1958).
30. B. CAPON AND R. L. FOSTER, *J. Chem. Soc. C.*, 1654 (1970).
31. T. OSAWA AND Y. NAKAZAWA, *Biochem. Biophys. Res. Commun.* **130**, 56 (1966).
32. A. F. HOLLEMAN AND G. WILHELMY, *Rec. Trav. Chim.* **21**, 432 (1902); N. V. SIDGWICK AND W. M. ALDOUS, *J. Chem. Soc.* **119**, 1001 (1921).
33. R. MELDOLA AND F. G. C. STEPHENS, *J. Chem. Soc.* **89**, 923 (1906).
34. F. BALLARDIE, B. CAPON, J. D. G. SUTHERLAND, D. COCKER, AND M. SINNOTT, *J. Chem. Soc., Perkin Trans. I*, 2418 (1973).
35. F. WEYGAND, *Methods Carbohyd. Chem.* **1**, 183 (1962).
36. R. U. LEMIEUX, T. L. NAGABHUSHAN, AND I. K. O'NEILL, *Canad. J. Chem.* **46**, 413 (1976).
37. L. F. FIESER AND M. FIESER, "Reagents for Organic Synthesis," p. 1292. Wiley, New York, 1967.
38. M. L. WOLFROM AND A. P. THOMPSON, *Methods Carbohyd. Chem.* **1**, 209 (1962).
39. J. FINDLAY, G. A. LEVVY, AND C. A. MARSH, *Biochem. J.* **69**, 467 (1958).
40. G. ZEMPLEN AND E. PACSU, *Ber.* **62**, 1613 (1929).
41. M. BERGMANN AND L. ZERVAS, Ber. **64**, 975 (1931).
42. H. J. BARBER, *J. Chem. Soc.*, 102 (1943).
43. A. B. FOSTER AND R. HEMS, *Methods Carbohyd. Chem.* **6**, 198 (1972).
44. Cf. H. B. SINCLAIR, *Carbohyd. Res.* **15**, 147 (1970).
45. C. J. MOREL, *Helv. Chim. Acta* **41**, 1501 (1958).
46. W. E. DEMING, "Statistical Adjustment of Data," Dover, New York, 1964; W. E. WENTWORTH, *J. Chem. Educ.* **42**, 96, 162 (1965).