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Studies on the Acceptor Specificity of the Lysozyme-Catalyzed Transglycosylation Reaction*

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ABSTRACT: Incubation of the bacterial cell wall tetrasaccharide, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc, with hen egg-white lysozyme in the presence of D-glucose, leads to the formation of GlcNAc-β-(1→4)-MurNAc-D-Glc and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-D-Glc. The effect of time and of concentration of D-glucose on the formation of the two new oligosaccharides was investigated. The results were accounted for by a tranglycosylation mechanism, in which the cell wall tetrasaccharide or the corresponding hexasaccharide formed from it, serve as donors of GlcNAc-β-(1→4)-MurNAc or of GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc residues, respectively, and D-glucose serves as an acceptor. The lysozyme-catalyzed transfer of GlcNAc-β-(1→4)-MurNAc residues from the cell wall tetrasaccharide to some 40 other mono- and disaccharides was investigated. With eleven of these, no transfer products could be detected, and they were classified as "nonacceptors," whereas the rest were found to be acceptors. The relative efficiency of the acceptors in the transfer reaction was determined by competition experiments, in which the cell wall tetrasaccharide was incubated with lysozyme and two different saccharides in equimolar concentration. Marked differences were found in the acceptor ability of these saccharides, and it was shown that the results obtained provide evidence for the existence of subsites *E* and *F* in the active site of lysozyme, as postulated by Phillips and his coworkers. *N*-Acetyl-D-glucosamine was the best acceptor among the monosaccharides tested, and it was concluded that it interacts with subsite *E* mainly through its 2-acetamido group; binding to subsite *E* through the 6-CH_2OH and 3-OH groups was also indicated. Disaccharides were found to be better acceptors than the corresponding monosaccharides, the best acceptors being GlcNAc-β-(1→4)-MurNAc and its methyl ester. The *N*-acetylmuramic acid residue in these acceptors appears to bind to the enzyme at subsite *F* and to form contacts through its 6-CH_2OH and the CH_3 of the lactyl moiety, but not through its 2-acetamido group.

All these and other findings reported are in agreement with the three-dimensional lysozyme–substrate model.

As a result of the X-ray crystallographic studies of Phillips and his coworkers, hen egg-white lysozyme became the first enzyme for which the three-dimensional structure was elucidated (Blake *et al.*, 1965, 1967a; Phillips, 1966, 1967; Johnson *et al.*, 1968). The structures of several lysozyme–inhibitor complexes have been also seen worked out by these investigators (Johnson and Phillips, 1965), and a model has been proposed by them for the lysozyme–substrate complex, as well as a mechanism for the action of the enzyme (Phillips, 1966, 1967; Blake *et al.*, 1967b). According to Phillips and his coworkers, the active site of lysozyme lies in a cleft on the surface of the enzyme. This cleft can accommodate six sugar residues, designated as A, B, C, D, E, and F, in corresponding subsites *A* to *F*. The positions of the first three subsites were located by X-ray studies of enzyme–inhibitor complexes. The presence of subsites *D*, *E*, and *F* was inferred by fitting molecular models of substrates to the three-dimensional model of the enzyme. It was further assumed that cleavage occurs between sugar residues D and E.

In contrast to our detailed knowledge of the structure of lysozyme, considerably less is known about its enzymatic properties (for a recent review, see Chipman and Sharon, 1969). The main reason for this is that the number and variety of well-defined substrates for the enzyme are very limited. The substrates are mainly β-1→4-linked oligosaccharides of *N*-acetylhexosamines derived either from chitin, with the

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general structure of $(GlcNAc)_n$,[1] or from bacterial cell walls, with the general structure $(GlcNA\text{-}\beta\text{-}(1\rightarrow4)MurNAc)_n$ (Sharon, 1967). Some oligosaccharides containing D-glucose (Zehavi *et al.*, 1968), and several glycosides of *N*-acetyl-D-glucosamine oligomers (Osawa, 1966; Osawa and Nakazawa, 1967; Zehavi and Jeanloz, 1968), have also been shown to serve as substrates for the enzyme. However, because of the difficulties involved in the synthesis of oligosaccharides in general, and of those of amino sugars in particular, no truly synthetic substrates for lysozyme have been prepared as yet, and studies of the enzyme have been carried out mostly with substrates derived from natural sources. The finding that lysozyme can catalyze transglycosylation, in addition to hydrolysis (Sharon and Seifter, 1964; Kravchenko and Maksimov, 1964), provided an important approach to the study of the mode of action and specificity of the enzyme. As the transfer reaction was shown to occur with a wide variety of acceptor saccharides (Pollock *et al.*, 1967a,b), the specificity requirements of the acceptor subsites in the active site of the enzyme (subsites *E* and *F*) could readily be investigated. Such studies are of special importance, since in this region binding of substrates or inhibitors has not been demonstrated by X-ray crystallography.

In this publication, we present data on the lysozyme-catalyzed transfer of GlcNAc-β-(1→4)-MurNAc residues from the cell wall tetrasaccharide GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc to a large number of mono- and disaccharide acceptors. The relative efficiency of various saccharides as acceptors was determined in competition experiments, in which the cell wall tetrasaccharide was incubated with lysozyme and two acceptor saccharides in equimolar concentration. The results obtained have provided evidence for the existence of subsites *E* and *F* in the active site of lysozyme. Taken in conjunction with the studies on the structure of products formed in the transfer reactions, our results have enabled us to draw certain conclusions on the binding of saccharides at subsites *E* and *F* and on the nature of the interactions which take place upon binding. These results are interpreted in terms of the three-dimensional structure of the lysozyme–substrate complex, as proposed by Phillips.

Experimental Section

Materials. Hen egg-white lysozyme (twice recrystallized, salt free) was a product of Worthington; lysostaphin (Browder *et al.*, 1965) was kindly given by Dr. P. A. Tavormina.

The cell wall tetrasaccharide, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc and the corresponding disaccharide, GlcNAc-β-(1→4)-MurNAc, were isolated from *Micrococcus lysodeikticus* as previously described (Sharon *et al.*, 1966; Sharon, 1967). Radioactive di- and tetrasaccharide were prepared from the corresponding unlabeled compounds by tritiation (Wilzbach, 1957) and were purified as described (Chipman *et al.*, 1968). The preparation of the monomethyl ester of the cell wall disaccharide has been reported (Chipman *et al.*, 1967). The trisaccharide, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc, was obtained by enzymatic degradation of the cell wall tetrasaccharide with lysostaphin (Leyh-Bouille *et al.*, 1966; Pollock and Sharon, 1969, 1970). GlcNAc-β-(1→4)-MurNAc-β-D-Glc and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-β-D-Glc were similarly prepared by a lysozyme-catalyzed transfer reaction (Pollock and Sharon, 1969; Pollock, 1969). These compounds contain 88% of β-1→4-linked D-glucose, and about 12% of the corresponding isomers which have a β-1→2-linkage between their *N*-acetylmuramic acid and D-glucose residues. *N*-Acetyl-D-glucosamine was a gift of Pfizer and Co.; it was recrystallized from ethanol–acetone to a constant melting point of 202–203°. Di-*N*-acetylchitobiose, GlcNAc-β-(1→4)-GlcNAc, was prepared from chitin (Rupley, 1964) and was further purified by paper chromatography. It was incubated with the cell wall tetrasaccharide and lysozyme to give GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc which was isolated by preparative paper electrophoresis (Pollock, 1969). Xylobiose was obtained from a partial acid hydrolysate of xylan (Tu, 1962; Bailey and Hassid, 1966). D-Glc-β-(1→4)-D-Xyl was a gift of Dr. James K. Alexander, GlcNAc-β-(1→4)-D-Gal was a gift of Dr. Aurelin Acher, and α-methyl and α-ethyl glycosides of *N*-acetyl-D-glucosamine a gift of Professor A. Neuberger. Benzyl 4-*O*-methyl-β-D-xylopyranoside was a gift of Dr. P. J. Garegg; it was catalytically hydrogenated with palladium to give syrupy 4-*O*-methyl-D-xylose which migrated upon paper chromatography in three solvent systems, as described in the literature (Hough and Jones, 1952).

Radioactive monosaccharides were purchased from the Radiochemical Center and from the New England Nuclear Corp. All other materials were of the highest purity available.

Counting of Radioactivity. Counting was done in Bray's dioxane scintillation solution (Bray, 1960), with a Packard Tri-Carb scintillation counter for 5 min. In experiments where only one of the compounds was labeled (either with ^3H or ^{14}C) counting was done in a single channel and the results were corrected for background only. In experiments with double labeling, ^{14}C and ^3H were simultaneously determined by double-channel counting, and the results obtained were corrected for background and for crossover between channels, but not for per cent efficiency. The specific activities of the compounds used in these experiments were determined in an identical manner, by double-channel scintillation counting of the complete reaction mixture, which contained the labeled compounds together with weighed quantities of nonradioactive carrier saccharides. Here again, corrections were made only for background and for crossover between channels. The experiments were designed so that the corrections for crossover were always small (less than 1% for ^3H to ^{14}C, and less than 12% for ^{14}C to ^3H).

Paper Electrophoresis. Electrophoresis was carried out for 90 min on Whatman No. 3MM paper under a constant voltage of 50 V/cm in a pyridinium acetate buffer (1.2 M pyridine adjusted with acetic acid to pH 6.5). The electrophoretograms were analyzed with a Vanguard gas-flow strip scanner to locate the radioactive peaks. For quantitative measurements, the strips were divided into sections of 3–20 mm, depending on the proximity of the peaks. The paper sections were then placed in counting vials and 0.6 ml of

[1] Abbreviations used are: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid (2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose); M_T, electrophoretic mobility relative to cell wall tetrasaccharide.

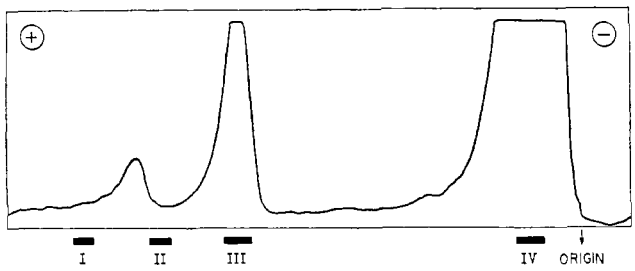


FIGURE 1: Radioactivity strip scan of an electrophoretogram of a reaction mixture after incubation with lysozyme for 24 hr at 37°. Incubation mixtures consisted of lysozyme (0.03 mg), unlabeled cell wall tetrasaccharide (1 μmole), and D-glucose-*U*-^{14}C (1 μmole) in 0.10 ml of ammonium acetate–acetic acid buffer (0.1 M in acetate) (pH 5.25). The solid bars denote the position of markers: (I) GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc, (II) GlcNAc-β-(1→4)-MurNAc, (III) GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc, and (IV) D-glucose. The small fast-running peak, between I and II, is GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-D-Glc, while the peak corresponding in electrophoretic mobility to marker III is GlcNAc-β-(1→4)-MurNAc-D-Glc.

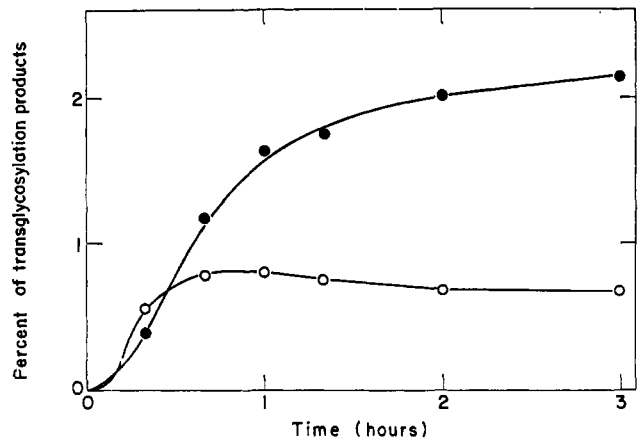


FIGURE 2: Rate of formation of new transfer products during lysozyme-catalyzed transglycosylation. Unlabeled tetrasaccharide (1 × 10^{-2} M) was incubated with lysozyme (1 mg/ml) at pH 5.25, 37° in the presence of D-glucose-*U*-^{14}C (1 × 10^{-2} M, 8 × 10^5 cpm). The total solution volume was 100 μl and at various time intervals over a period of 3 hr, aliquots (10 μl) were analyzed by paper electrophoresis at pH 6.5 for 1.5 hr. The radioactive peaks corresponding in electrophoretic mobility (see Figure 1) to D-glucose, GlcNAc-β-(1→4)-MurNAc-β-D-Glc (●—●) and GlcNAcβ-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-β-D-Glc (○—○), were quantitated by cutting sections of the electrophoretogram and counting in Bray's scintillation solution. The per cent of transglycosylation products refers to the mole per cent of tetrasaccharide converted into the products. Since equimolar concentrations of D-glucose and tetrasaccharide were used, the per cent of each transfer product was obtained by calculating the percentage of radioactivity represented by the transfer product in relation to the total radioactivity per electrophoretic strip.

H_2O was added to each vial. After the strips had been allowed to stand overnight, dioxane scintillation solution (Bray, 1960) was added, and the samples were counted as described above. GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc, and the cell wall di- and tetrasaccharides were used as markers. The unlabeled markers were revealed by the sodium hydroxide reagent (Sharon and Seifter, 1964).

Transglycosylation Experiments. CONCENTRATION DEPENDENCE. Reaction mixtures (100 μl) consisted of a particular concentration of D-glucose-*U*-^{14}C (1 × 10^{-2}–5 × 10^{-1} M containing approximately 8 × 10^5 cpm), unlabeled tetrasaccharide (1 μmole), and lysozyme (0.1 mg) in ammonium acetate–acetic acid buffer (0.1 M in acetate), pH 5.25. Incubation was at 37° and aliquots (10–30 μl) were withdrawn from the samples at different time intervals and spotted on paper for electrophoresis. The radioactive peaks corresponding in electrophoretic mobility to D-glucose and to the two transfer products GlcNAc-β-(1→4)-MurNAc-D-Glc (M_T 0.68) and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-D-Glc (M_T 0.88) were cut up into sections and counted by liquid scintillation in Bray's solution.

SURVEY OF ACCEPTORS. In order to ascertain which compounds could serve as acceptors in the lysozyme-catalyzed transglycosylation reaction, saccharides were incubated with the donor, the cell wall tetrasaccharide, in the presence of lysozyme, at a molar ratio of acceptor to donor of 50 to 1. Typical reaction mixtures consisted of 80 μl of an aqueous solution of the tritium-labeled tetrasaccharide (specific activity 1 × 10^5 cpm/μmole, 15 μmoles/ml), 20 μl of a solution of lysozyme (1.5 mg/ml) in ammonium acetate–acetic acid buffer (0.5 M in acetate), pH 5.25, to which potential acceptor (60 μmoles) was added. Reaction mixtures were incubated at 37° and aliquots (30 μl) were removed immediately after addition of lysozyme (zero time), at 12 and 24 hr for analysis by paper electrophoresis. The total radioactivity per strip was about 30,000 cpm.

COMPETITION EXPERIMENTS. In a typical competition experiment, 100 μl of a solution of tritium-labeled tetrasaccharide (specific activity 1 × 10^5 cpm/μmole, 15 μmoles/ml) was added to 100 μl of ammonium acetate buffer (pH 5.25) which contained 60 μg of lysozyme and an equimolar concentration of two acceptor saccharides. The final concentration of tetrasaccharide was thus 7.5 × 10^{-3} M and that of lysozyme, 0.3 mg/ml. The reaction mixture was incubated at 37° and at various time intervals usually up to 24 hr, aliquots (30 μl) were withdrawn for analysis by paper electrophoresis.

In cases where the new compounds formed had similar electrophoretic mobilities, competition experiments were carried out by using unlabeled tetrasaccharide (1 × 10^{-2} M) and two acceptor saccharides labeled with different radioisotopes, ^{14}C and ^{3}H in equimolar concentration (2.5 × 10^{-2}–12.5 × 10^{-2} M). In the region of the electrophoretogram corresponding to GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc, ^{14}C and ^{3}H were simultaneously determined by double-channel scintillation counting and from the number of counts (corrected for blanks and for crossover between channels) and the specific activities of the acceptors, determined in the same way, the ratio of products was calculated.

Results

Experiments with D-*Glucose.* Incubation of D-glucose-*U*-^{14}C with the cell wall tetrasaccharide and lysozyme leads to the formation of two transfer products (Figure 1) which have been characterized mainly as GlcNAc-β-(1→4)-Mur-

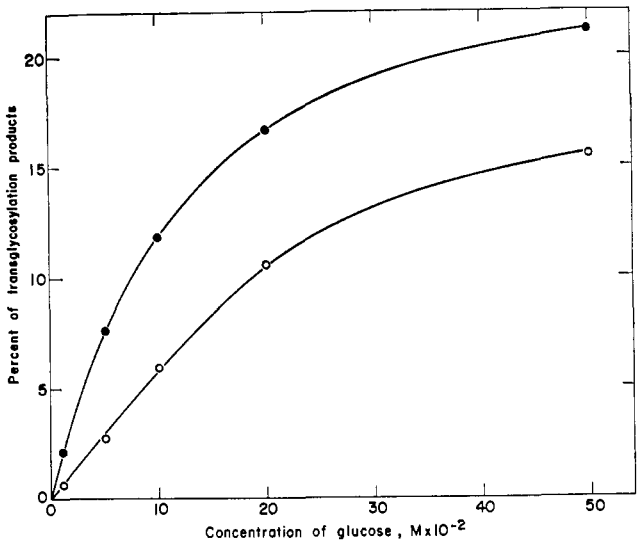


FIGURE 3: Dependence of transglycosylation on the concentration of D-glucose. Reaction mixture as in Figure 2, except that concentration of D-glucose was varied. After 6-hr incubation, a 30-μl aliquot was analyzed by paper electrophoresis. The radioactive peaks corresponding in electrophoretic mobility (see Figure 1) to D-glucose, GlcNAc-β-(1→4)-MurNAc-β-D-Glc (O—O), and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-β-D-Glc (●—●) were quantitated. The per cent of translgycosylation products refers to the mole per cent of tetrasaccharide converted into these products. Calculations are based on percent of total radioactivity and are adjusted for the different molar concentrations of D-glucose used in the individual experiments.

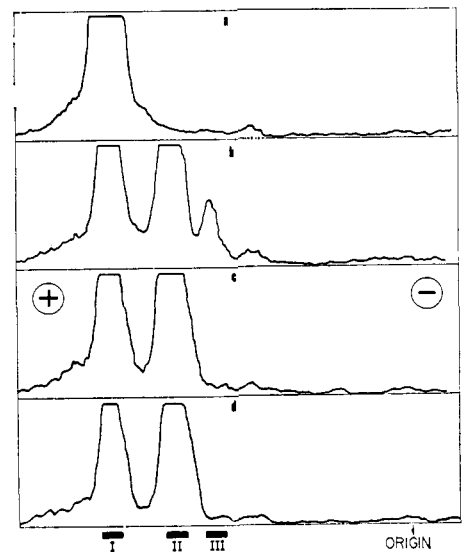


FIGURE 4: Paper electrophoretic strip scans of reaction mixtures containing potential acceptor and cell wall tetrasaccharide at a molar ratio of 50 to 1, after 12-hr incubation (for experimental details, see text): (a) tetrasaccharide only, without lysozyme; (b) tetrasaccharide with lysozyme and L-fucose; (c) tetrasaccharide with lysozyme and L-mannose; and (d) tetrasaccharide with lysozyme and 2-deoxy-D-galactose. The solid bars denote the position of markers: (I) GlcNAc β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc, (II) GlcNAc-β-(1→4)-MurNAc, and (III) GlcNAc-β-(1→4)-MurNAc-D-Glc.

NAc-β-(1→4)-D-Glc and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-β-(1→4)-D-Glc with some β-1→2-linked D-glucose (Pollock, 1969; Pollock and Sharon, 1969). We did not succeed in separating the saccharides with 1→4- and 1→2-linked D-glucose, in any of the paper chromatography or paper electrophoresis systems used (Pollock, 1969; Pollock and Sharon, 1969).

When equimolar concentrations (1×10^{-2} M) of D-glucose and tetrasaccharide are used, the extent of transfer to form the glucose-containing trisaccharide and the glucose-containing pentasaccharide is very small (Figure 2). The quantity of pentasaccharide increases rapidly at first, but reaches a plateau at about 0.5–1 hr, whereas the rate of formation of the trisaccharide starts to level off somewhat later on. When the concentration of tetrasaccharide (1×10^{-2} M) is kept constant and that of D-glucose is increased from 1×10^{-2} to 5×10^{-1} M, the extent of transfer as measured after 6-hr incubation increases markedly (Figure 3).

Survey of Acceptors. Some forty saccharides were tested for their ability to serve as acceptors of GlcNAc-β-(1→4)-MurNAc residues from the tritium-labeled cell wall tetrasaccharide in the lysozyme-catalyzed transfer reaction, using an acceptor to donor ratio of 50:1. As a result of these experiments, the compounds tested were divided into two classes.

ACCEPTORS: for which at least 5% of the total radioactive counts were found in the form of newly synthesized transfer products. A typical result in this class with L-fucose as acceptor is shown in Figure 4b.

NONACCEPTORS: in which no significant number of counts (less than 0.2% of the total) was found in the region corresponding to new transfer products (see Figure 4c,d, for results of experiments with L-mannose and 2-deoxy-D-galactose). The nonacceptors are listed in Table I. Since with D-glucose

TABLE I: Saccharides Which Do Not Serve as Acceptors ("Nonacceptors") in the Lysozyme-Catalyzed Transglycosylation Reaction.[a]

Monosaccharides	Disaccharides
1. L-Man	1. D-Glc-α-(1→4)-D-Glc
2. L-Rha	2. D-Gal-β-(1→4)-D-GlcNAc
3. D-Rib	
4. D-GalNAc	
5. 2-Deoxy-D-Gal	
6. Me-β-D-Gal	
7. D-Glc-6-PO_4	
8. α-D-Glc-1-PO_4	
9. Me-α-D-Glc	

[a] Substrate, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-*U-t*, 1.2×10^{-2} M. Saccharide, 60×10^{-2} M; lysozyme 0.3 mg/ml; pH 5.25. Incubation was carried out at 37° up to 24 hr, and aliquots were analyzed by paper electrophoresis as described in text. For the compounds listed, no formation of new saccharides, in addition to the products of digestion of the cell wall tetrasaccharide, could be detected. The upper limit for transfer to the nonacceptors, relative to D-glucose, is 1%.

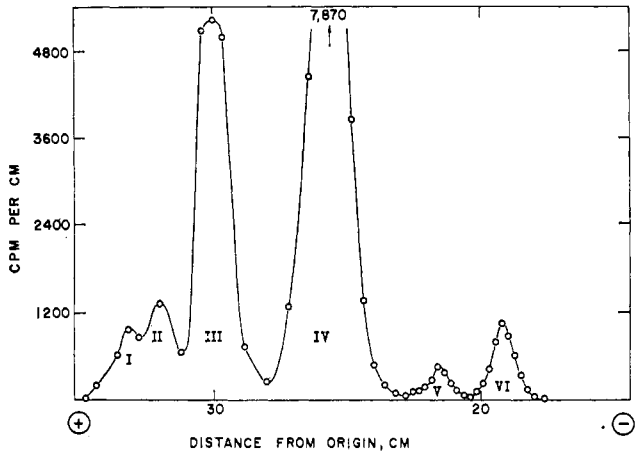


FIGURE 5: Paper electrophoresis at pH 6.5 of a reaction mixture after 4-hr incubation at 37° consisting of tritium-labeled tetrasaccharide (7.5×10^{-3} M), equimolar concentration (1.5×10^{-3} M) of GlcNAc and GlcNAc-β-(1→4)-GlcNAc and hen egg-white lysozyme (0.3 mg/ml) in pH 5.25 ammonium acetate–acetic acid buffer (0.1 M in acetate). Sections (1 cm or less) from the electrophoretogram were cut up and counted by liquid scintillation: (I) cell wall octasaccharide, (II) cell wall hexasaccharide, (III) cell wall tetrasaccharide, (IV) cell wall disaccharide, GlcNAc-β-(1→4)-MurNAc, (V) GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc, and (VI) GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc.

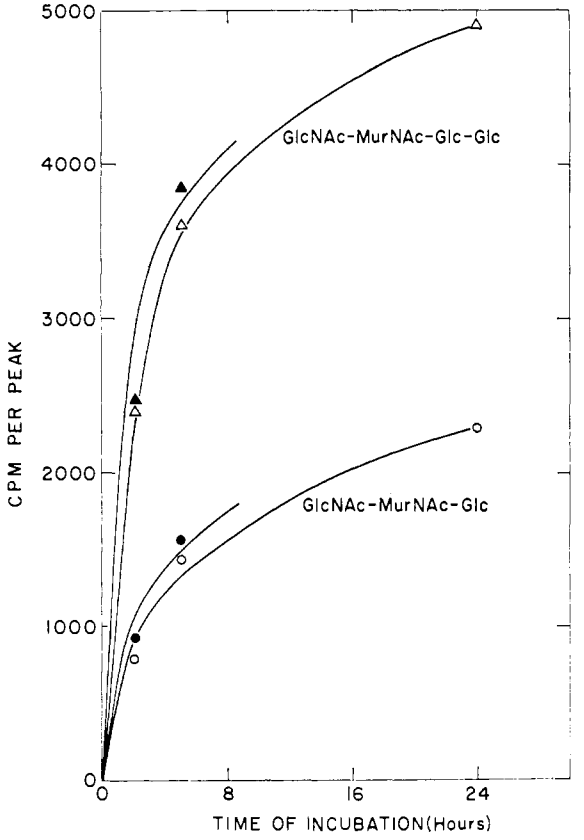


FIGURE 6: Formation of GlcNAc-β-(1→4)-MurNAc-D-Glc and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-D-Glc-β-(1→4)-D-Glc in lysozyme-catalyzed transglycosylation. Incubation mixtures contained tritium-labeled tetrasaccharide (7.5×10^{-3} M), equimolar concentrations of D-glucose and cellobiose, lysozyme (0.3 mg/ml) in pH 5.25 ammonium acetate–acetic acid buffer (0.1 M in acetate); they were analyzed by paper electrophoresis as described in the text. Open symbols, concentration of each of the acceptors 7.5×10^{-2} M. Closed symbols, 12.5×10^{-2} M. Circles, GlcNAc-β-(1→4)MurNAc-D-Glc; triangles, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-D-Glc-β-(1→4)-D-Glc.

under the same conditions, 20% of the counts was found in the trisaccharide region, the upper limit of transfer to the nonacceptors, relative to D-glucose, is 1%. All acceptors were further tested in competition experiments.

Competition Experiments. WITH LABELED TETRASACCHARIDE. Figure 5 shows the distribution of radioactivity on an electrophoretogram of a reaction mixture containing GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-*U*-*t* with an equimolar mixture of GlcNAc-β-(1→4)-GlcNAc (X_1) and *N*-acetyl-D-glucosamine (X_2), after 4-hr incubation with lysozyme. The change in the quantities of products with time in two different experiments in which D-glucose and D-Glc-β-(1→4)-D-Glc were used as acceptors is presented in Figure 6. Since the acceptors were used at equimolar concentrations, the ratio of the products of transfer is equal to the ratio of the total counts in the corresponding peaks. These ratios are presented in Figure 7, in which it is seen that they are reproducible within ±5%, and that they are not constant but decrease with time. The ratio of GlcNAc-β-(1→4)-MurNAc-X_1 (tetrasaccharide) to GlcNAc-β-(1→4)-MurNAc-X_2 (trisaccharide) formed decreased as incubation proceeded both when D-Glc-β-(1→4)-D-Glc and D-glucose, and when GlcNAc-β-(1→4)-GlcNAc and *N*-acetyl-D-glucosamine were used as acceptors. By plotting the ratio values against time of incubation, straight lines were obtained which could be extrapolated to zero time (Figure 7).

The results of a large series of competition experiments, carried out in a similar way, but using different pairs of mono- and disaccharides, are summarized in Table II. The transfer product ratio, in all cases, was obtained by extrapolation to zero time, as described in Figure 7.

LABELED ACCEPTORS. Where tritiated tetrasaccharide was the donor, it was not possible to compare directly the acceptor efficiencies of two different neutral monosaccharides, since trisaccharide-transfer products are obtained with the same electrophoretic mobility (Pollock, 1969; Pollock and Sharon, 1969). Such comparisons could, however, be carried out using unlabeled tetrasaccharides and two acceptors labeled with different radioisotopes. For example, the results of comparisons of D-glucose-6-*t* with D-mannose-*1*-^{14}C as acceptors are given in Figure 8. It can be seen that the number of counts in the radioactive peaks corresponding to trisaccharides labeled with tritium and ^{14}C increased with time and gradually leveled off. In this experiment, the ratio between products did not change with time. The ratio of counts of ^{3}H to counts of ^{14}C in the region of migration of new trisaccharides was 1.41 after 2-hr incubation, 1.49 after 4 hr, 1.49 (6 hr), and 1.50 (24 hr), the average value being 1.47. Since the ratio of specific activities of the D-glucose-*6*-*t* to the D-mannose-*1*-^{14}C, in the reaction mixture (which was measured under the same conditions), was 1.45, the ratio of transfer products formed, GlcNAc-β-(1→4)-MurNAc-X_1 to GlcNAc-β-(1→4)-MurNAc-X_2 is 1.0. The reproducibility in these experiments was usually within ±5%.

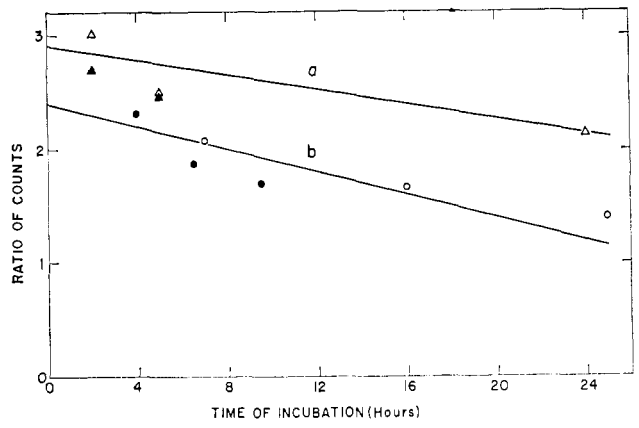


FIGURE 7: Ratio of transfer products formed during lysozyme-catalyzed transglycosylation reactions. Incubation mixtures contained tritium-labeled tetrasaccharide (7.5×10^{-3} M), equimolar concentrations of either D-glucose and cellobiose, or *N*-acetyl-D-glycosamine and GlcNAc-β-(1→4)-GlcNAc, and lysozyme (0.3 mg/ml) in pH 5.25 ammonium acetate–acetic acid buffer (0.1 M in acetate). Aliquots were analyzed by paper electrophoresis. "Ratio of counts" means number of counts of new tetrasaccharide transfer product divided by the corresponding number of the new trisaccharide transfer product. Concentrations of acceptor saccharides: (1) (△—△) D-glucose and cellobiose at 7.5×10^{-2} M; (▲—▲) D-glucose and cellobiose at 12.5×10^{-2} M. (b) (○—○, ●—●) Two experiments with *N*-acetyl-D-glucosamine and GlcNAc-β-(1→4)-GlcNAc at 1.5×10^{-3} M.

The results of this and other experiments carried out in a similar manner are summarized in Table III.

Discussion

Transfer to D-*Glucose and Transglycosylation Scheme.* Incubation of the cell wall tetrasaccharide, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc, with

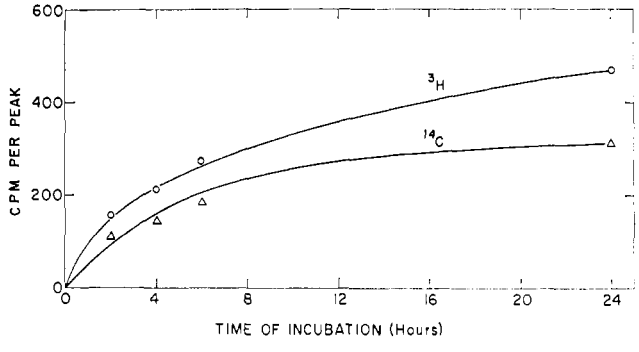


FIGURE 8: Formation of trisaccharides during a lysozyme-catalyzed transglycosylation reaction. A mixture containing unlabeled cell wall tetrasaccharide (1×10^{-2} M), and an equimolar concentration (12.5×10^{-2} M) of D-glucose-6-*t* (1.46×10^4 cpm/μmole) and D-mannose-*1*-^{14}C 1.01×10^4 cpm/μmole and hen egg-white lysozyme (0.3 mg/ml) in ammonium acetate–acetic acid buffer (0.1 M in acetate)(pH 5.25) was incubated at 37° for a period of 24 hr. Aliquots were analyzed by paper electrophoresis as described in text. Measurements of radioactivity were all done by double-channel scintillation counting, and corrected for blanks and crossover between channels only. (○—○) GlcNAc-β-(1→4)-MurNAc-D-Glc; (△—△) GlcNAc-β-(1→4)-MurNAc-D-Man.

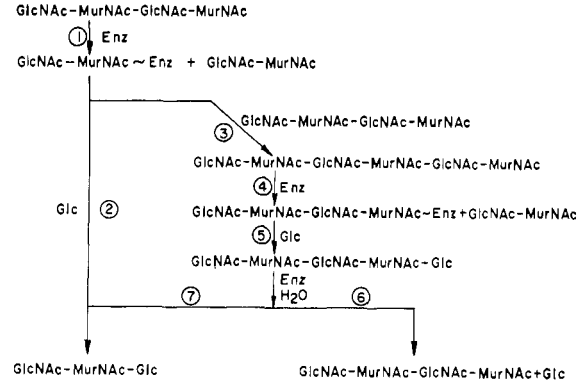


FIGURE 9: Scheme for the lysozyme-catalyzed formation of the trisaccharide GlcNAc-β-(1→4)-MurNAc-D-Glc and the pentasaccharide GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-D-Glc from the tetrasacchride GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc and D-glucose. Enz, enzyme.

lysozyme in the presence of D-glucose leads to the formation of new transfer products (Figure 1), characterized as GlcNAc-β-(1→4)-MurNAc-D-Glc and GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-D-Glc where the linkage between MurNAc and D-Glc is predominantly β-1→4 together with a small amount (about 12%) or corresponding isomer possessing the β-1→2 linkage (Pollock, 1969; Pollock and Sharon, 1969). The formation of these transfer products can readily be accounted for by a transglycosylation scheme given in Figure 9.

The first step in this scheme (reaction 1) is the formation of a glycosyl-enzyme intermediate GlcNAc-β-(1→4)-MurNAc~E, with the concomitant release of the GlcNAc-β-(1→4)-MurNAc from the reducing half of the substrate molecule. In the glycosyl-enzyme intermediate, the activated GlcNAc-β-(1→4)-MurNAc is bound at subsites *C* and *D*, with the reducing end at *D*. The intermediate can then react with water (reaction not included in Figure 9) to yield the disaccharide GlcNAc-β-(1→4)-MurNAc (hydrolysis); with glucose to form GlcNAc-β-(1→4)-MurNAc-β-D-Glc (reaction 2) or with another molecule of tetrasaccharide to yield the corresponding hexasaccharide (reaction 3; Chipman *et al.*, 1968). The hexasaccharide will then react, through a new glycosyl-enzyme intermediate, to form the pentasaccharide GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-β-D-Glc (reactions 4 and 5). From the latter compound, the trisaccharide GlcNAc-β-(1→4)-MurNAc-β-D-Glc can be formed, either by hydrolysis (one molecule, by reaction 7), or by transfer to glucose (two molecules). In all the transfer reactions from the glycosyl-enzyme intermediates, β-D-linked saccharides are formed, showing that there is overall retention of configuration of the glycosidic bond (Pollock, 1969; Pollock and Sharon, 1969).

As shown in Figure 3, the quantity of pentasaccharide stops increasing quite early. This may be ascribed to its digestion by lysozyme (Figure 9, reactions 6 and 7) which is assumed to be considerably faster than the digestion of the corresponding trisaccharide. The general pattern of the formation and cleavage of the pentasaccharide is similar to that previously observed by us in the case of higher cell wall oligosaccharides produced by transglycosylation reactions

TABLE II: Competition Experiments to Measure the Efficiency of Various Saccharides as Acceptors in the Lysozyme-Catalyzed Transglycosylation Reaction Carried Out with Radioactive Labeled Tetrasaccharide.

Expt No.[a]	Acceptors		Concn (M)	Ratio[b]
	X_1	X_2		
1[c]	D-Glc-β-(1→4)-D-Glc	D-Glc	7.5×10^{-2}	2.9
2[c]			12.5×10^{-2}	
3[c]	GlcNAc-β-(1→4)-GlcNAc	GlcNAc	1.5×10^{-3}	2.4
4	GlcNAc-β-(1→4)-GlcNAc	D-Glc	7.5×10^{-2}	10.0
5	D-Glc-β-(1→4)-D-Glc	GlcNAc	3.5×10^{-2}	0.7
6	GlcNAc-β-(1→4)-MurNAc Me ester	GlcNAc	1.0×10^{-2}	3.3
7	GlcNAc-β-(1→4)-D-Gal	GlcNAc	5.2×10^{-2}	1.6
8	D-Xyl-β-(1→4)-D-Xyl	D-Xyl	3.8×10^{-2}	1.5
9	D-Glc-β-(1→4)-D-Xyl	D-Glc	8.0×10^{-2}	1.4
10	D-Glc-β-(1→4)-D-Glc	Me-β-6-deoxy-D-Glc	7.5×10^{-2}	2.1
11	D-Glc-β-(1→4)-D-Glc-β-(1→4)-D-Glc	D-Glc-β-(1→4)-D-Glc	7.0×10^{-2}	0.5
12	D-Glc-β-(1→4)-D-Glc	D-ManNAc	7.5×10^{-2}	5.9
13	D-Glc-β-(1→4)-D-Glc	L-Fuc	7.5×10^{-2}	5.7
14	D-Glc-β-(1→4)-D-Glc	L-Glc	7.5×10^{-2}	5.9
15	D-Glc-β-(1→4)-D-Glc	D-Fuc	7.5×10^{-2}	6.9
16	D-Glc-β-(1→4)-D-Glc	D-Lyx	7.5×10^{-2}	4.4
17	D-Glc-β-(1→4)-D-Glc	L-Xyl	7.5×10^{-2}	4.3
18	D-Glc-β-(1→4)-D-Glc	L-Ara	7.5×10^{-2}	6.1
19	D-Glc-β-(1→4)-D-Glc	D-Ara	7.5×10^{-2}	7.0
20	D-Glc-β-(1→6)-D-Glc	D-Glc	7.5×10^{-2}	1.8

[a] A description of the technique used in these experiments is given in the text. Briefly, tritium-labeled cell wall tetrasaccharide (7.5×10^{-3} M) was incubated at pH 5.25 and 37° with lysozyme and an equimolar mixture of two acceptor saccharides, at the concentration listed. At various time intervals, usually up to 24 hr, aliquots were analyzed by paper electrophoresis at pH 6.5. [b] The ratio given is that of new products formed (GlcNAc-MurNAc-X_1:GlcNAc-MurNAc-X_2) obtained by extrapolation to zero time, as described in Figure 7. [c] Details of these experiments are given in Figures 6 and 7. Reproducibility of ratios was within ±5%.

during the lysozyme digestion of GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc (Chipman *et al.*, 1968).

When the concentration of D-glucose was increased, the quantity of transfer products also increased (Figure 4). At the highest concentration of D-glucose used, 20 mole % of the tetrasaccharide were converted into GlcNAc-β-(1→4)-MurNAc-D-Glc and 15 mole % into GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc-β-(1→4)-MurNAc-D-Glc. According to the transglycosylation scheme (Figure 9), 1 mole of tetrasaccharide is required for the formation of 1 mole of trisaccharide and 2 moles for the formation of 1 mole of pentasaccharide. Thus, with 0.5 M D-glucose, 50 mole % of the tetrasaccharide substrate was used up. One reason for the incomplete conversion of the tetrasaccharide into transfer products is that, in addition to transglycosylation, concomitant hydrolysis takes place.

Survey of Acceptors. For surveying a large number of saccharides as possible acceptors in the transglycosylation reaction, a high ratio of the potential acceptor to the donor tetrasaccharide (50:1) was used, and the reaction mixtures examined at zero time and after 12- and 24-hr incubation. Only products which were formed by transfer of a disaccharide moiety to the acceptor could be detected by this method since products formed by transfer of a tetrasaccharide moiety (see scheme, Figure 9) could not be separated from the cell wall disaccharide on paper electrophoresis at pH 6.5.

In some cases, the new trisaccharides and tetrasaccharides formed were isolated on a preparative scale, and their structure established by the use of chemical and enzymatic techniques. These included, in addition to D-glucose, the products of transfer to *N*-acetyl-D-glucosamine, D-galactose, D-xylose (Pollock and Sharon, 1969, 1970), and D-Glc-β-(1→4)-D-Glc, and GlcNAc-β-(1→4)-GlcNAc (Zehavi *et al.*, 1968). In other cases, identification was based on the rate of migration on paper electrophoresis, which is identical for all trisaccharides (M_T 0.68) or tetrasaccharides (M_T 0.60) formed by transfer to neutral saccharide acceptors.

Many of the compounds tested were found to serve as acceptors. These included monosaccharides, not only of the D configuration, but also of the L configuration, and disaccharides as well as some higher oligosaccharides. Where a saccharide served as an acceptor, the newly formed trisaccharide represented at least 5% of the total radioactivity present on the electrophoretic strip under the assay conditions (Figure 4). Where a saccharide was not considered an acceptor, no radioactivity above background (less than 0.2% of total counts) was present in the area corresponding to new transfer products. The nonacceptors are listed in Table I.

TABLE III: Competition Experiments to Measure the Efficiency of Various Saccharides as Acceptors in the Lysozyme-Catalyzed Transglycosylation Reaction, Carried Out with Radioactive Acceptors.[a]

Expt No.	Acceptors		Concn (M)	Ratio[b]
	X_1	X_2		
1	D-Man-^{14}C	D-Glc-*t*	12.5×10^{-2}	1.0
2	GlcNAc-^{14}C	D-Glc-*t*	2.5×10^{-2}	4.5
3	D-Xyl-^{14}C	D-Glc-*t*	12.5×10^{-2}	0.9
4	3-*O*-Me-D-Glc-^{14}C	D-Glc-*t*	12.5×10^{-2}	0.4
5	2-Deoxy-D-Glc-^{14}C	D-Glc-*t*	12.5×10^{-2}	1.0
6	Me-β-D-Glc-^{14}C	D-Glc-*t*	12.5×10^{-2}	1.0
7	Me-α-D-Glc-^{14}C	D-Glc-*t*	12.5×10^{-2}	0
8	D-Gal-^{14}C	D-Glc-*t*	12.5×10^{-2}	0.5
9[c]	GlcNAc-^{14}C	GlcNAc-β-(1→4)-MurNAc-*t*	3.5×10^{-2}	0.3

[a] Cell wall tetrasaccharide (1×10^{-2} M) was incubated at pH 5.25, 37°, with lysozyme (0.3 mg/ml) and an equimolar mixture of two radioactive saccharides, one labeled with ^{3}H and the other with ^{14}C. At different time intervals, up to 24 hr, aliquots were analyzed by paper electrophoresis at pH 6.5. In the region of the electrophoretogram corresponding to the newly formed trisaccharides, in expt 1–8, ^{3}H and ^{14}C were simultaneously determined by means of double-channel scintillation counting, and from the number of counts and the specific activities of the acceptors (counted in the same way), the ratio of the products (GlcNAc-MurNAc-X_1:GlcNAc-MurNAc-X_2) was calculated. ^{14}C saccharides used were labeled at their C-1 poistion; the glucose was labeled at the hydrogen linked to C-6, whereas the cell wall disaccharide was uniformely labeled. [b] Average of at least three measurements reproducible within ±5%. No change with time of incubation was observed, except in expt 9 (see below). [c] In this experiment, ratio of products is based on extrapolation to zero time of incubation (see Table II and Figure 8). The peak areas which contain ^{3}H correspond to (GlcNAc-β-(1→4)-MurNAc)$_n$, where n = 1, 2, 3, or 4; only the peak corresponding to n = 2 was used for calculation of the product ratio.

Comparison of Acceptors. The relative efficiency of the acceptors was compared by competition experiments in which either labeled tetrasaccharide was incubated with two unlabeled acceptors, or where the acceptors were labeled with two different radioisotopes and the tetrasaccharide was unlabeled. In both types of experiment, the two acceptors were in equimolar concentrations. This technique allows the direct comparison of acceptors, such as *N*-acetyl-D-glucosamine and GlcNAc-β-(1→4)-GlcNAc, which are also inhibitors of lysozyme (Sharon, 1967), with acceptors such as D-glucose and D-Glc-β-(1→4)-D-Glc which are not inhibitors. The results of the competition experiments are given in Tables II and III. When the two acceptors X_1 and X_2 are monosaccharides, the ratio of products was found to be constant throughout the period of incubation (Figure 8), as the newly formed trisaccharides are cleaved by the enzyme at a very slow rate (Pollock and Sharon, 1969, 1970). However, when X_1 is a disaccharide, and X_2 a monosaccharide, the ratio of transfer products decreases as incubation proceeds (Figure 7), probably because the newly formed tetrasaccharide is cleaved by the enzyme faster than the corresponding trisaccharide. One reason for the difference in rates of cleavage of tetrasaccharide and trisaccharide transfer products may be attributed to the fact that the tetrasaccharides form less stable nonproductive complexes with the enzyme than the corresponding trisaccharides (Pollock, 1969). In experiments of this type, we have therefore extrapolated the ratio of transfer products to zero time. It should be noted, however, that because of the differences in rates of hydrolysis just mentioned, the ratios obtained in the comparisons of disaccharide with monosaccharides may in most cases be lower than the "true" ratios of rates of transfer to these compounds. It is difficult to give estimates for the possible discrepancy between our experimental results and the true values, especially in view of the complexity of the reactions catalyzed by lysozyme.

The ratios given in Tables II and III are those found in experiments in which equimolar concentrations of two acceptors were used. The same ratios are also obtained by indirect comparison of different sets of experiments. Thus in expt 3, Table II, it is found that [GlcNAc-MurNAc-*GlcNAc*-*GlcNAc*]/[GlcNAc-MurNAc*GlcNAc*] = 2.4 (in this and the following equations the acceptor portions of the transfer products are in italic type).

From expt 4 in the same table [GlcNAc-MurNAc-*GlcNAc*-*GlcNAc*]/[GlcNAc-MurNAc-*Glc*] = 10.0; therefore, [GlcNAc-MurNAc-*GlcNAc*]/[GlcNAc-MurNAc-*Glc*] = 10/2.4 = 4.2.

A similar value (4.1) is obtained for the ratio of *N*-acetyl-D-glucosamine and D-glucose as acceptors by comparison of expt 1 and 2 with expt 5 in Table II. This value is in very good agreement with that obtained by direct comparison of the two acceptors (expt 2, Table III).

It is clear from the results of the above comparisons that the ratio of transfer products is not affected by whether the acceptors tested are inhibitors of lysozyme or not. Furthermore, although the effect of varying acceptor concentration on the ratio of products was not investigated in detail, the above results strongly suggest that these ratios are independent of acceptor concentration.

The product ratios given in Tables II and III can thus be combined and summarized in a single table (Table IV) which grades the various saccharides according to their

TABLE IV: Ability of Various Saccharides to Serve as Acceptors in the Lysozyme-Catalyzed Transglycosylation Reaction.

Compd	Saccharide	Rel Ability[a] to Serve as an Acceptor (D-Glc = 100)	Linkage	
			Established[b]	Suggested[c]
1	GlcNAc-β-(1→4)-MurNAc	1480		1→4
2	GlcNAc-β-(1→4)-MurNAc Me ester	1480		1→4
3	GlcNAc-β-(1→4)-GlcNAc	1080		1→4
4	GlcNAc-β-(1→4)-D-Gal	720		1→4
5	D-GlcNAc	450	1→4	
6	D-Glc-β-(1→4)-D-Glc	280		1→4
7	D-Glc-β-(1→4)-D-Glc	180		1→4
8	D-Glc-β-(1→4)-D-Glc-β-(1→4)-Glc	140		1→4
9	D-Glc-β-(1→4)-D-Xyl	140		1→4
10	D-Xyl-β-(1→4)-Xyl	135		1→4, 1→3
11	Me-β-6-deoxy-D-Glc	135		1→4
12	D-Glc	100	1→4, 1→2	
13	D-Man	100		1→4
14	2-Deoxy-D-Glc	100		1→4
15	Me-β-D-Glc	100		1→4
16	D-Xyl	90	1→2, 1→3, 1→4	
17	L-Xyl	65		1→2, 1→3
18	D-Lyx	65		1→4
19	D-Gal	50	1→2	
20	L-Fuc	50		1→2
21	L-Glc	50		1→2, 1→3
22	D-ManNAc	50		1→4
23	L-Ara	45		1→2
24	D-Ara	40		1→2
25	D-Fuc	40		1→2
26	3-*O*-Me-D-Glc	40		1→4, 1→2
27	Me-α-D-GlcNAc	(40)		1→4
28	Et-α-D-GlcNAc	(40)		1→4
29	4-*O*-Me-D-Xyl	(35)		1→2, 1→3

[a] The figures for **1–26** are derived from the competition experiments (Tables II and IV) as described in the text. For **27–29** the figures are based not on competition experiments, but on experiments carried out as used for the survey of acceptors: the number of counts in the region of the newly formed trisaccharide GlcNAc-β-(1→4)-MurNAc-X, was compared to that found with D-glucose under the same conditions. [b] Pollock and Sharon (1969, 1970). [c] See text. In cases where the suggested linkage is not discussed in text, it is based on analogy with closely related compounds.

ability to serve as acceptors in the lysozyme-catalyzed transglycosylation reaction. In this table we have chosen arbitrarily the value of 100 for D-glucose, and have used this value as a basis for comparison with the other compounds tested. We have also listed the linkages formed, some of which have been established by analysis of the products (Pollock and Sharon, 1969, 1970).

Evidence for Subsites E and F. The data in Table IV allow us to make important conclusions on the specificity requirements of lysozyme for acceptors. Since different saccharides differ in their acceptor ability, this clearly shows that the enzyme possesses at least one acceptor subsite, which may be identical with subsite *E* proposed by Phillips and his colleagues (Blake *et al.*, 1967b; Phillips, 1966, 1967). Furthermore, as disaccharides are better acceptors than the corresponding monosaccharides, the existence of an additional acceptor site on the enzyme, subsite *F*, also proposed by Phillips (Blake *et al.*, 1967b; Phillips, 1966, 1967), is clearly indicated (Pollock *et al.*, 1967).

It is of interest to note here that although subsites *E* and *F* have not been seen in the crystal, other evidence supports their existence. The chitin oligomers $(GlcNAc)_n$ are cleaved by lysozyme at rates which increase as n increases from 3 to 6 (Rupley, 1967). The cell wall hexasaccharide $(GlcNAc\text{-}\beta\text{-}(1\rightarrow4)\text{-}MurNAc)_3$ is cleaved some 5000 times faster than the corresponding tetrasaccharide under the same experimental conditions (Chipman *et al.*, 1968; Chipman and Sharon, 1969). However, the cell wall octasaccharide $(GlcNAc\text{-}\beta\text{-}(1\rightarrow4)\text{-}MurNAc)_4$ is digested at the same rate as the hexasaccharide (Chipman *et al.*, 1968), which is to be expected since it can only occupy the same six subsites (*A* to *F*).

In the discussion which follows, it is assumed that once saccharides are bound to the enzyme in the acceptor sub-

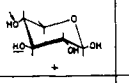
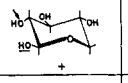
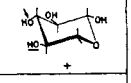
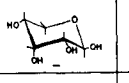
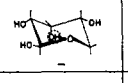
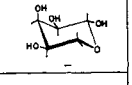
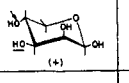
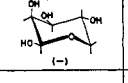
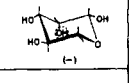
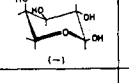
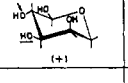
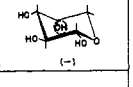
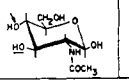
COMPOUND	FORMULA	LINKAGE		
		1→4	1→2	1→3
D-XYLOSE		 +	 +	 +
D-RIBOSE		 −	 −	 −
D-LYXOSE		 (+)	 (−)	 (−)
D-ARABINOSE		 (−)	 (+)	 (−)
N-ACETYL D-GLUCOSAMINE				

FIGURE 10: Four pentoses tested as acceptors in the lysozyme-catalyzed translgycosylation reaction. The conformational structures, in their most stable forms, are drawn in their probable orientation at substite *E*, so as to show their relationship to *N*-acetyl-D-glucosamine bound at this subsite. The arrow denotes the oxygen involved in the formation of the glycosidic bond. +, linkage formed; −, not formed. When the + and − symbols are in parentheses, no experimental evidence on the linkage formed is available, and its nature is only suggested (for detailed discussion, see text).

sites, they attack the activated intermediate equally well. Although the rate-limiting step is very likely among the reactions leading to the formation of the glycosyl-enzyme intermediate (see, for example, Johnson *et al.*, 1968), the proportions of products formed in the competition experiments will depend on the concentration of the acceptors at subsite *E* (or *E* and *F*). In other words, the differences in acceptor ability are a result of different and specific binding at subsites *E* (or *E* and *F*), prior to nucleophilic attack of the acceptor on the glycosyl-enzyme intermediate. For simplicity, differences in nucleophilic reactivity of the hydroxyls which react with the glycosyl-enzyme intermediate are neglected, whether they are at the same ring position in different saccharides, or not. This assumption is justified since the attacking groups are all hydroxyls linked to carbon atoms of pyranose rings, and also because no difference has been observed in the acceptor ability of various alcohols and phenols which differ markedly in their nucleophilicity (Rupley *et al.*, 1968).

Interaction of the 2-Acetamido Group with Subsite E. Since *N*-acetyl-D-glucosamine is a much better acceptor than D-glucose, it may be concluded that the acetamido group plays an important role in the binding at subsite *E*. We can estimate the approximate contribution of the acetamido group to the free energy of binding at subsite *E* from the relative ability of these two saccharides to serve as acceptors. For this purpose, only the formation of β-1→4-linked products is taken into consideration, and it is assumed that the conformation of the enzyme upon binding *N*-acetyl-D-glucosamine at subsite *E* is identical with that which binds D-glucose. In the case of transfer to *N*-acetyl-D-glucosamine the newly formed bond is solely β-1→4, whereas in the transfer to D-glucose, the same bond is found only in about 90% of the product (Pollock and Sharon, 1969). For the same reaction, *N*-acetyl-D-glucosamine as an acceptor is therefore five times better than D-glucose. Hence $\Delta F_{GlcNAc} - \Delta F_{Glc} = -RT \ln (K_1/K_2) = -1.0$ kcal/mole, where ΔF_{GlcNAc} and ΔF_{Glc} are the free energies of binding to subsite *E* of *N*-acetyl-D-glucosamine and of D-glucose, respectively, K_1 and K_2 are their binding constants and K_1/K_2 is taken to equal 5, the corrected product ratio.

When the equatorial acetamido group at C-2 of *N*-acetyl-D-glucosamine is replaced by an OH, either equatorial (D-glucose) or axial (D-mannose), no interaction of this group with subsite *E* takes place, as D-glucose, D-mannose, and 2-deoxy-D-glucose are equally efficient as acceptors. The finding that *N*-acetyl-D-mannosamine is one-half as good an acceptor as D-glucose seems to be an example of steric crowding, resulting from the presence of the bulky axial 2-acetamido in this amino sugar.

Our evidence on the interaction of the acetamido group at subsite *E* with the enzyme supports the proposal of Phillips and his coworkers (Blake *et al.*, 1967b), based on three-dimensional model building, that the CONH part of this group is linked by hydrogen bonds to the main peptide chain of lysozyme: the NH group to the main-chain carbonyl of Glu-35, and the CO to the β-NH_2 of Asn-44.

Interaction of the 6-CH_2OH and the 3-OH with Subsite E. Conclusions about interactions with the enzyme of substituents attached at other ring positions of the pyranose residue bound at subsite *E* are not as straightforward as for the case of the 2-acetamido group. Comparison of the results obtained with D-glucose and D-xylose as acceptors, would suggest no role for the 6-CH_2OH of the D-glucose in binding to subsite *E*. Both sugars are almost exclusively in their pyranose form, have the same conformation (C-1), and contain the same proportions of anomers (~63% of β) (Angyal, 1969). However, if only β-1→4-linked products are taken into consideration, D-glucose appears to be two times better an acceptor than D-xylose, since only half of the trisaccharides formed with D-xylose is 1→4, whereas in the case of D-glucose, the 1→4-linked trisaccharide accounts for about 90% of the products (Pollock and Sharon, 1969). A role for the 6-CH_2OH in binding to the enzyme at subsite *E* is supported by the finding that 6-deoxy-D-glucose is a somewhat better acceptor than D-glucose. The replacement of an OH group at the 6-CH_2OH by an hydrogen may result in better hydrophobic contacts with the enzyme, possibly with the side chain of Val-109, as can be seen from examination of the lysozyme–substrate model. A calculation similar to that made for the contribution of the 2-acetamido group to the free energy of binding, gives a value of about −0.4 kcal/mole for the contribution of the 6-CH_2OH (formation of 1→4-linked D-glucose trisaccharide as compared to formation of 1→4-D-xylose trisaccharide).

The role of the OH group at C-3 may be assessed from a comparison of the pentose acceptors tested, taking into consideration the types and proportions of linkages formed (see Figure 10). Thus, D-xylose will fit into subsite *E* in a form which is superimposed on that of *N*-acetyl-D-glucosamine in the proposed three-dimensional enzyme–substrate model. This mode of interaction results in the formation of a 1→4 linkage. In addition, in its β form, D-xylose may bind at the same subsite in a form which results from rotation of the ring clockwise by 120° in its plane, to give 1→2 linkage, or by turning over the ring to give a 1→3 linkage.

D-Ribose is not an acceptor (Table I) and with the aid of Figure 10, where it is drawn in its most stable conformation (β, C-1), it is possible to explain this finding. This pentose cannot bind in subsite *E* in the same manner as *N*-acetyl-D-glucosamine because it has no groups which can make contact with the enzyme: it lacks both an acetamido group at carbon-2 and a 6-CH_2OH group. Furthermore, its 3-OH is axial and it would appear that this group must be equatorial to interact with the enzyme; therefore, D-ribose cannot form a 1→4 bond. Assuming that it can interact with the enzyme in a different way, a 1→3 bond can be excluded on the grounds that the 3-OH is axial and cannot act as a nucleophile. The reason why D-ribose does not form a 1→2 linkage is less clear. It is true that by rotating the D-ribose molecule clockwise by 120°, its 2-OH will be positioned for nucleophilic attack and its 1-OH (in the β form) will be superimposed on the 3-OH of *N*-acetyl-D-glucosamine forming a contact with the enzyme. However, in this rotated form (Figure 10), its axial 3-OH will occupy the position of the 5-H of *N*-acetyl-D-glucosamine and may strongly interfere with binding. Indeed, careful examination of the model shows that this axial hydroxyl is pointed too far in toward the enzyme.

The results obtained with D-xylose and D-ribose strongly suggest therefore that for a pentose to be an acceptor, it must fit into subsite *E* so that in positions corresponding to the C-3 and C-4 of *N*-acetyl-D-glucosamine in this subsite, there are two diequatorial hydroxyls situated in trans positions. One of these, that superimposed on the 4-OH of *N*-acetyl-D-glucosamine in the three-dimensional lysozyme–substrate model, serves for bond formation, whereas the other, superimposed on the 3-OH of *N*-acetyl-D-glucosamine, binds the acceptor to the enzyme. This supports the suggestion of Phillips that the 3-OH at subsite *E* makes a polar interaction with the γ-CO of Glu-57 of the enzyme.

Both D-lyxose (β, C-1) and D-arabinose (β, C-1) will satisfy the requirements of two trans diequatorial hydroxyls, the former by forming a 1→4 linkage, and the latter at 1→2 linkage (Figure 10). Although no experimental evidence is available for the linkages formed by these two sugars, they are about half as good acceptors as D-xylose (Table IV), in agreement with the above suggestion that they form only one type of linkage.

Preliminary experiments seem to support the suggestion that in the case of pentopyranoses a pair of vicinal trans diequatorial hydroxyls may be the minimal requirement for their serving as acceptors. In these experiments we have examined the lysozyme-catalyzed transfer of a GlcNAc-β-(1→4)-MurNAc moiety from the cell wall tetrasaccharide to 1,2-*cis*-cyclohexanediol, and to 1,2-*trans*-cyclohexanediol, under the conditions used for the survey of acceptors (see Experimental Section). It was found that the trans compound was an acceptor, whereas the cis compound was not. It should be recalled that Rupley and his coworkers (Rupley *et al.*, 1968) have found that cyclohexanol is a very poor acceptor, not much better than water (a nonacceptor, in our classification). 3-*O*-Methyl-D-glucose is an acceptor, possibly because the methyl group can form some hydrophobic contacts with the enzyme, or perhaps because it forms a 1→2 linkage only.

Formation of a 1→2 Linkage with D-*Galactose.* An examination of the three-dimensional lysozyme–substrate model

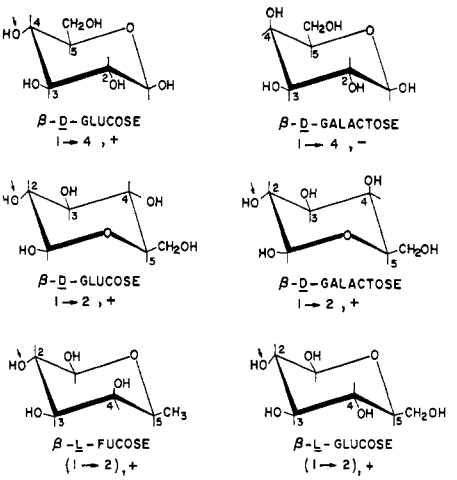


FIGURE 11: Four hexoses shown to be acceptors in the lysozyme-catalyzed transglycosylation reaction. The conformational structures in their most stable forms are drawn in their probable orientation at substite *E*, with (1→4)-linked D-glucose occupying the subsite in an orientation in which its ring atoms are superimposed on the corresponding atoms of *N*-acetyl-D-glucosamine at the same subsite. The arrow denotes the oxygen involved in the formation of the new glycosdic bond. +, linkage formed; −, not formed. When the linkages are in parentheses, no experimental evidence on their nature is available (for detailed discussion, see text).

indicates that if D-galactose is to occupy subsite *E* in the same orientation as *N*-acetyl-D-glucosamine (Figure 11) then its 4-OH, which is axial, would point out away from carbon-1 of the *N*-acetylmuramic acid residue at subsite *D*. Formation of a β-1→4 linkage would thus not be possible. However, our results (Pollock and Sharon, 1969, 1970) show that the 2-OH of D-galactose acts as the nucleophile to form GlcNAc-β-(1→4)-MurNAc-β-(1→2)-D-Gal. In fact, monosaccharides related to D-galactose which lack the 2-OH such as 2-deoxy-D-galactose and *N*-acetyl-D-galactosamine are not acceptors (Table I). A different positioning of D-galactose in subsite *E* must therefore be assumed. It can be achieved by 120° clockwise rotation by the sugar in the plane of the ring (Figure 11). As a result of this movement, the D-galactose ring will fit snugly into subsite *E*, and will utilize its anomeric OH, in the β configuration, for a polar contact with the enzyme. In this new orientation, the anomeric hydroxyl will occupy the position of the 3-OH of *N*-acetyl-D-glucosamine in subsite *E*. If the hydrogen of this hydroxyl is removed and replaced by methyl or saccharide residues as in methyl β-D-galactoside or *N*-acetyllactosamine (D-Gal-β-(1→4)-GlcNAc), no transfer is observed (Table I). Furthermore, in the rotated form of D-galactose, the 6-CH_2OH group will be directed into subsite *F*, where it can easily be accommodated by the enzyme. Rotation by 120° of the D-galactose ring brings its axial 4-OH to a position which now lies over the ring oxygen of *N*-acetyl-D-glucosamine (Figure 11). Again, examination of the model of the enzyme shows that this axial group would not make contact with the enzyme. Moreover, since D-fucose and L-arabinose also have an axial 4-OH and are similar in structure and conformation to D-galactose, it becomes very likely that they too will form only 1→2 linkages in the transfer reaction.

Like D-galactose, D-glucose can also rotate by 120° (Figure

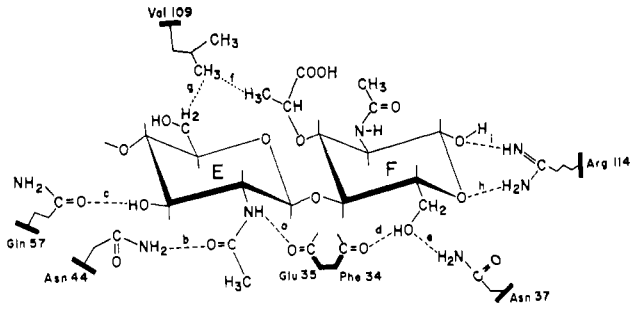


FIGURE 12: Contacts formed between lysozyme and GlcNAc-β-(1→4)-MurNAc bound at subsites *E* and *F*. (-----) Polar contacts; (· · · ·) nonpolar contacts. Contacts a–e have been proposed by Phillips and his coworkers on the basis of model building, and evidence to support their proposal is presented in the text. Contacts f and g have been proposed by us on the basis of the experimental evidence described in this paper and inspection of the lysozyme–substrate model. No evidence is available for contacts h and i, also proposed on the basis of model building.

11) to give some GlcNAc-β-(1→4)-MurNAc-β-(1→2)-D-Glc (Pollock and Sharon, 1969). Other sugars, such as D-mannose or 2-deoxy-D-glucose will not form 1→2 linkages because their 2-OH is either axial or absent. As shown in Figure 11, L-glucose and L-fucose can be accommodated in subsite *E* in an orientation which may lead to the formation of 1→2-linked transfer products.

Although the above considerations can explain most of the results with monosaccharide acceptors and nonacceptors, some findings seem to be unaccounted for. Thus, it is not clear why methyl α-D-glucoside is not an acceptor, whereas closely related α-D-glycosides such as the methyl and ethyl glucosides of *N*-acetyl-D-glucosamine (Table IV, **27** and **28**), and p-nitrophenyl α-D-glucoside (Raftery and Rand-Meir, 1968), are acceptors.

Specificity of Subsite F. In all cases, disaccharides were found to be better acceptors than the corresponding monosaccharides, implicating the presence of an additional subsite, *F*, adjacent to subsite *E*. Since D-Glc-β-(1→6)-D-Glc is a better acceptor than D-glucose, and GlcNAc-β-(1→4)-D-Gal a better acceptor than *N*-acetyl-D-glucosamine, it appears that subsite *F* is rather spacious. This is in accord with the results of X-ray crystallography. From the findings that GlcNAc-β-(1→4)-GlcNAc is almost four times better an acceptor than D-Glc-β-(1→4)-D-Glc, it is evident that differences in the observed transfer product ratio can be explained entirely by the 2-acetamido group at subsite *E*; therefore, the 2-acetamido group at subsite *F* apparently does not contribute significantly to interaction with the enzyme.

A further analysis of subsite *F* suggests a role for the 6-CH_2OH group since the presence of a D-xylose residue at this subsite instead of a D-glucose residue lowers the relative acceptor ability by one-half (compare D-Glc-β-(1→4)-D-Glc with D-Glc-β-(1→4)-D-Xyl in Table IV). However, the value obtained for D-Xyl-β-(1→4)-D-Xyl is too high (it should be nearer to 100 in Table IV) unless the unlikely assumption is made that with this compound as an acceptor, linkages other than 1→4 are formed. It should be noted that interaction of the 6-CH_2OH at subsite *F* with the enzyme has in fact been proposed by Phillips and his coworkers.

Since GlcNAc-β-(1→4)-MurNAc and its methyl ester are better acceptors than GlcNAc-β-(1→4)-GlcNAc, it may be assumed that the lactyl moiety of *N*-acetylmuramic acid interacts with the enzyme at subsite *F*. Examination of the lysozyme–substrate model leads us to believe that the interaction is between the methyl group of the lactyl moiety of *N*-acetylmuramic acid and a methyl group of Val-109.

The total contribution of a *N*-acetylmuramic acid residue to the free energy of binding at subsite *F*, obtained by comparing the acceptor ability of GlcNAc-β-(1→4)-MurNAc with that of *N*-acetyl-D-glucosamine (1480 as compared to 450, Table IV) is −0.7 kcal/mole. This value is most probably too low, in view of the possibility, mentioned earlier, that ratios obtained by us are lower than the "true" ratios of rates of transfer (because of the faster rate of hydrolysis of tetrasaccharides as compared to trisaccharides). Adding this value to those obtained for subsite *E* (−1.0 kcal/mole for the 2-acetamido group and −0.4 kcal/mole for the 6-CH_2OH), a total of −2.1 kcal/mole is obtained which is a lower limit for the contribution of different functional groups described in this paper to binding at the acceptor subsites of the enzyme. The relation of this value to the total free energy of binding at subsites *E* and *F*, as obtained by other techniques, has been discussed in detail elsewhere (Chipman and Sharon, 1969). The various contacts formed between the best acceptor, GlcNAc-β-(1→4)-MurNAc (also part of the natural substrate of lysozyme), and the enzyme, based on the three-dimensional enzyme–substrate model of Phillips, are summarized in Figure 12.

Acknowledgments

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Solid-Phase Synthesis of [4-Threonine]-oxytocin. A More Potent and Specific Oxytocic Agent Than Oxytocin*

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ABSTRACT: As part of a study concerned with identifying a postulated evolutionary intermediate between the 4-glutamine- and 4-serine-containing neurohypophysial hormones, [4-threonine]-oxytocin has been synthesized and tested for some of the pharmacological activities characteristic of the neurohypophysial hormones. The synthesis was accomplished by means of the Merrifield solid-phase method as employed for the synthesis of oxytocin and [8-phenylalanine]-oxytocin. After purification by gel filtration on Sephadex G-15, the product was obtained in 33% overall yield. [4-Threonine]-oxytocin has an oxytocic potency on the rat uterus of approximately 900 units/mg, twice the potency of oxytocin. It has a fowl vasodepressor potency of approximately 1480 units/mg, over three times the potency of oxytocin and has a milk-ejection potency of approximately 540 units/mg, about 20% greater then that of oxytocin. On the other hand, it possesses a rat vasopressor potency of approximately 0.43 unit/mg, only one-tenth the potency of oxytocin and a rat antidiuretic potency of approximately 3 units/mg, about half the potency of oxytocin. It may thus have a clinical application as a highly selective oxytocic agent.

The naturally occurring neurohypophysial hormones which have been characterized to date contain either a glutamine residue or a serine residue in position 4 of the common cyclic octapeptide structure. A mutation from serine to glutamine requires two base changes in the parent mRNA codons for these amino acids and this would seem to indicate the presence in nature of a hitherto uncharacterized 4-substituted intermediate as the "missing link" between the 4-serine- and 4-glutamine-containing neurohypophysial hormones. We have been concerned with trying to identify such an intermediate analog *via* synthetic means and have previously reported the pharmacological characteristics of three 4-proline-substituted analogs of the neurohypophysial hormones (Sawyer *et al.*, 1969). These analogs were found to possess very low activities in the standard assay systems and thus would be extremely difficult to detect in posterior pituitary extracts by the techniques currently in use.

In an extension of these studies, we now wish to report the synthesis and some pharmacological properties of [4-threonine]-oxytocin, an analog of oxytocin in which the glutamine residue in position four is replaced by a threonine

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