On Distinguishing between Possible Mechanistic Pathways during Lysozyme-Catalyzed Cleavage of Glycosidic Bonds*

M. A. Raftery and T. Rand-Meir

ABSTRACT: It has been shown that when lysozyme-catalyzed glycosidic bond cleavage of chitobiose is carried out in the presence of methanol transglycosylation yields methyl β-*N*-acetyl-D-glucopyranoside without formation of significant amounts of methyl α-*N*-acetyl-D-glucopyranoside. Thus, retention of configuration occurs at the glycosidic carbon (C_1) of the disaccharide during catalysis and eliminates the possibility of a single-displacement mechanism for the enzyme. Use has been made of this transglycosylation reaction to enzymatically synthesize oligomeric *p*-nitrophenyl β-D-glucosaminides from chitotetraose, *p*-nitrophenyl β-D-glucosaminide, and lysozyme. Such saccharides serve as substrates for lysozyme with release of *p*-nitrophenol. Similar synthesis of oligosaccharide nitrophenyl glycosides from chitotetraose, *p*-nitrophenyl β-D-glucoside, and lysozyme has been carried out. Since lysozyme effects hydrolysis of the glycosidic bonds in such oligosaccharides, anchimeric assistance of an *N*-acetyl group has been eliminated as a necessary pathway for the enzyme-catalyzed cleavage of glycosidic bonds. Similar enzymatic synthesis of an oligosaccharide substrate from chitotetraose and *p*-nitrophenyl 2-deoxy-β-D-glucoside has eliminated the necessity of invoking a mechanism involving acetamido or C-2 oxyanion participation by substrate due to general base catalysis by the enzyme.

In the system used the relative rates of lysozyme-catalyzed hydrolysis of the β-(1–4)-*p*-nitrophenyl glycosides of *N*-acetyl-D-glucosamine, D-glucose, and 2-deoxy-D-glucose were 2:1:16. Only two closely related possibilities remain to explain the mechanistic pathway traversed by these substrates during catalysis by lysozyme.

With the recent elucidation of the structure of lysozyme in the crystalline state by use of X-ray analysis techniques (Blake *et al.*, 1965, 1967a) and the parallel studies in the same laboratory of the interactions between the enzyme and various inhibitors (Johnson and Phillips, 1965; Blake *et al.*, 1967b; Phillips, 1967), widespread interest has been aroused pertaining to the precise mechanism of action of lysozyme. Owing to the stability of the crystalline lysozyme–inhibitor complexes studied by Blake *et al.*, it was obvious that the catalytic groups of the enzyme were not encompassed by these small molecules. On the basis of the known nonproductive interactions between lysozyme and chitotriose and further fitting of sugar rings as extensions of the observed complex, it has been suggested (Blake, 1966; Blake *et al.*, 1967a,b) that the most reasonable explanation of the catalytic activity of lysozyme is that two carboxyl groups (aspartic acid residue 52 and glutamic acid residue 35) which are close to the inhibitor binding site function as acid and base to effect hydrolysis of susceptible glycosidic bonds. Owing to the established mechanism of nonenzymatic glycoside hydrolysis in acidic medium (for a review of such nonenzymatic reactions, see Schalegar and Long, 1963; Cordes, 1967), it was suggested (Blake *et al.*, 1965, 1967a,b; Phillips, 1967; Vernon, 1967) that a similar mechanistic pathway involving an intermediate carbonium ion could plausibly function in the lysozyme-catalyzed hydrolysis of glycosidic bonds. Such a carbonium ion could be formed, it was argued, following protonation of a glycosidic oxygen by an acid group such as a protonated carboxyl side chain on the enzyme.

Since other mechanistic pathways are also plausible in such an enzymatic reaction catalyzed in the pH range 2–10, we have attempted to distinguish between several of these possibilities. In the present communication we present evidence which eliminates some of the possible mechanistic pathways.

Materials and Methods

Lysozyme was purchased from Sigma Chemical Co. (lot no. 668–8590). Chitin oligosaccharides obtained from acid hydrolysis of the polysaccharide (Rupley, 1964) were fractionated as described elsewhere (Raftery *et al.*, 1968). Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside was synthesized by previously published procedures (Zilliken *et al.*, 1957). Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside was purchased from Pierce Chemical Co. It was freed from a 10% contamination by the corresponding β-glycoside by chromatography on charcoal–Celite columns (Zilliken *et al.*, 1957). *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, *p*-nitrophenyl β-D-glucoside, and *p*-nitrophenyl β-D-galactoside were purchased from Cyclo Chemical Co. Pierce Chemical Co. was the source of

* Contribution No. 3655 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91109. *Received March 18, 1968.* Supported by the National Institutes of Health, U. S. Public Health Service (GM-14452-01) and the Irma Hoefly Fund for cancer research.

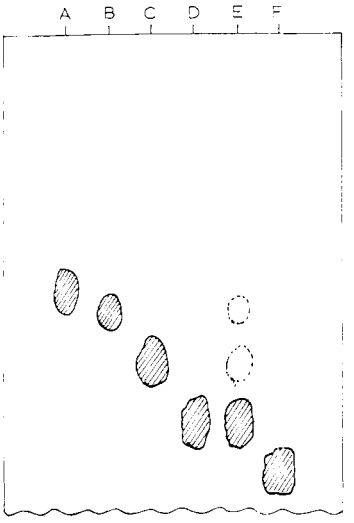

FIGURE 1: Tracing of paper chromatographic separation of transfer products obtained from lysozyme-catalyzed cleavage of chitobiose in the presence of methanol. (A) Chitobiose standard; (B) methyl β-chitobioside standard; (C) NAG standard; (D) methyl-β-NAG standard; (E) mixture from the transfer reaction; (F) methyl-α-NAG standard.

p-nitrophenyl β-D-xyloside, and *p*-nitrophenyl α-D-glucoside was obtained from Sigma Chemical Co. *p*-Nitrophenyl 2-deoxy-β-D-glucoside was synthesized from 2-deoxy-D-glucose (T. Rand-Meir and M. A. Raftery, unpublished data). Bio-Gel P-2 (200–400 mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif.

Partitioning by Methanol during Lysozyme Hydrolysis. Chitobiose (40 mg) and lysozyme (40 mg) were dissolved in 1.0 ml of 0.1 M citrate buffer (pH 5.5) which was 5 M with respect to methanol. A drop of toluene was added and the stoppered mixture was incubated at 40° for 16 hr. At the end of this time a small amount of precipitate was removed, after dilution of the mixture to 15 ml with water, by filtration through a Millipore filter (45-μ pore size). Lysozyme was quantitatively removed from the filtrate by ultrafiltration through a Diaflo membrane (UM-1) using a Diaflo ultrafiltration apparatus (Amicon Corp.). Analysis of the ultrafiltrate for protein by the Lowry procedure (Lowry *et al.*, 1951) revealed that all of the lysozyme had been retained by the membrane. The citrate buffer was removed by treatment of the ultrafiltrate with 2 g of Amberlite MB-1 resin. This treatment also rid the solution of free sugars, *i.e.*, those with reducing groups, leaving only the glycoside(s) formed. The treated solution was then lyophilized.

Paper chromatography was performed on Whatman No. 1 paper using pyridine–ethyl acetate–water (1:2:2) (upper phase) as the developing solvent. The solvent was allowed to drip off the paper during the development period of 16–18 hr. After thorough drying of the paper the separated saccharides were located using a modification (W. J. Dreyer, personal communication) of the chlorine peptide-bond spray (Mazur *et al.*, 1962).

Lysozyme Cleavage of p-Nitrophenyl N-Acetyl-β-D glucosaminides. Stock solutions of lysozyme (1.6×10^{-3} M) and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (1×10^{-1} M) in 0.1 M citrate buffer (pH 5.5) which contained 10% v/v *p*-dioxane were made up freshly prior to use. A stock solution of chitotetraose (1.2×10^{-2} M) was also made up in the same buffer. Aliquots of these solutions were mixed to yield final concentrations of enzyme equal to 4×10^{-4} M and *p*-nitrophenyl-NAG[1] equal to 5.0×10^{-2} M. In those experiments where chitotetraose was included, its final concentration was equal to 6×10^{-3} M, the final concentrations of enzyme and *p*-nitrophenyl-NAG being 4×10^{-4} and 2.5×10^{-2} M, respectively. Mixtures were incubated at 40° in stoppered tubes which contained a drop of toluene. Estimation of released *p*-nitrophenol was performed by adding aliquots (100 μg) of the incubation mixtures to 3.0 ml of 0.1 M potassium tetraborate (pH 9.0) and, following centrifugation, reading the absorbance of the resulting solution at 400 mμ in a Cary Model 14 spectrophotometer. A molar extinction coefficient for *p*-nitrophenol of 18,000 was used for quantitative estimation.

Lysozyme Hydrolysis of Other p-Nitrophenyl Glycosides. Incubation mixtures in 0.1 M citrate (pH 5.5) (10% *p*-dioxane, v/v) contained lysozyme (4×10^{-4} M), *p*-nitrophenyl glycoside (5×10^{-3}–5×10^{-1} M), and chitotetraose (6×10^{-3} M). The same method as outlined for *p*-nitrophenyl-NAG was used to determine the *p*-nitrophenol released. For effects of pH on the rate of release of *p*-nitrophenol, 0.1 M citrate buffers were used in the pH range 2–6 and 0.1 M citrate–phosphate buffers in the pH range 6–8 (McIlvaine, 1921).

Gel Filtration of Reaction Mixtures. Aliquots (usually 0.5 ml) of reaction mixtures of lysozyme, chitotetraose, and the various *p*-nitrophenyl monosaccharides were applied, following incubation at 40°, to a column (1.0×100 cm) of Bio-Gel P-2 (200–400 mesh) which had been equilibrated with 0.1 M NaCl solution. The same solvent was used as the eluting buffer. The flow rate of the column was 20.0 ml/hr and 1.0-ml fractions were collected. Protein was determined in the effluent by the method of Lowry *et al.* (1951). The *p*-nitrophenyl glycosides were detected by reading the optical density of each fraction at 280 mμ in a Cary 14 spectrophotometer.

Results

The Anomeric Form of the Product of Lysozyme Hydrolysis. Since two recent reports (Rupley and Gates, 1967; Dahlquist *et al.*, 1968) indicated that lysozyme can cleave chitobiose slowly to yield *N*-acetyl-D-glucosamine (as well as higher oligosaccharides as a result of transglycosylation), we have used this system to allow methanol to partition with water during the enzymatic hydrolysis and have identified the product of the reaction. As expected, some methyl glucosaminide (approximately 1–2 mg from 40 mg of chitobiose) was formed. Figure 1 shows a paper chromatogram of the

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *p*-nitrophenyl-NAG, *p*-nitrophenyl 2-deoxy-2-acetamido-β-D-glucosamine; β-methyl-NAG, methyl-2-deoxy-2-acetamido-β-D-glucosaminide; α-methyl-NAG, methyl-2-deoxy-2-acetamido-α-D-glucosaminide; NAM, *N*-acetylmuramic acid.

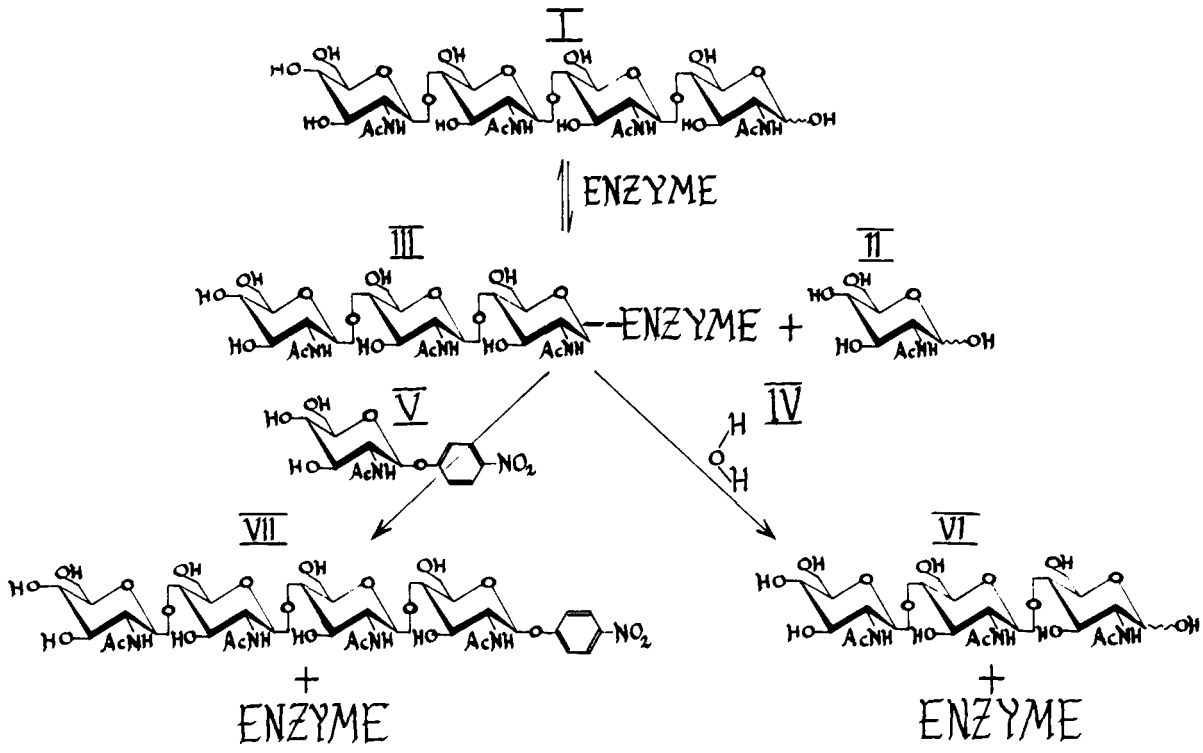

FIGURE 2: Scheme for the synthesis of oligomeric *p*-nitrophenyl glycosides from chitotetraose and *p*-nitrophenyl 2-acetamido-2-dexoy-β-D-glucopyranoside by lysozyme-mediated glycosyl transfer. The scheme suggests that chitotetraose (I) cleavage by lysozyme results in formation of free NAG (II) and a chitotriose–enzyme complex (III), which can on reaction with water (IV) give enzyme and free chitotriose (VI) or with another acceptor such as *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (V) give enzyme and *p*-nitrophenyl β-chitotetraoside. Other related products are also possible, depending upon which bond in the tetrasaccharide (I) is cleaved by the enzyme.

incubation mixture after being worked-up as described in the experimental section. All of the glycoside formed corresponded to methyl *N*-acetyl-β-D-glucosaminide. No trace of the corresponding α-glycoside was seen. This result agrees with a previous claim by Chipman *et al.* (1968). These investigators showed that treatment of a cell wall tetrasaccharide with lysozyme resulted in transglycosylation to form higher molecular weight products which were, however, not homogeneous as judged by paper chromatography. These were, after purification, susceptible to hydrolysis by the enzyme (yielding mainly the di- and tetrasaccharides) and were therefore considered to have all glycosidic bonds in the β configuration. However, in the absence of knowledge regarding the ability of lysozyme to hydrolyze or synthesize (*via* transglycosylation) α-linked glycosidic bonds this result must be regarded as suggestive of, rather than proof that, cleavage of glycosidic bonds by the enzyme proceeds with *complete* retention of configuration. Another report also agrees with our present findings. Rupley and Gates (1967) have stated that retention of configuration also obtains with a small molecule such as methanol. No data were given, however, and the extent of retention was not reported. This transglycosylation reaction has been further studied in a quantitative manner (Dahlquist *et al.*, 1968) using ^{14}C-labeled chitobiose and is has been shown that the β configuration is retained in the formation of β-methyl-NAG to an extent of at least 99.7%.

Lysozyme Cleavage of p-Nitrophenyl N-Acetyl-β-D-glucosaminides and Related Compounds. Lysozyme does not catalyze the hydrolysis of *p*-nitrophenyl β-D-glucosaminide at any observable rate. Several investigators have synthesized *p*-nitrophenyl β-D-chitobioside (Osawa, 1966; Lowe, 1967a,b; T. Rand-Meir, F. W. Dahlquist, and M. A. Raftery, unpublished data) and *p*-nitrophenyl β-D-chitotrioside (Osawa and Nakasawa, 1966) in an attempt to obtain a synthetic substrate for the enzyme. The results have been disappointing, since release of *p*-nitrophenol mediated by lysozyme is very slow. These results are understandable in light of the recent studies in this laboratory (Dahlquist *et al.*, 1966) and elsewhere (Rupley *et al.*, 1967) which indicate three contiguous strong binding sites on the enzyme which give rise to nonproductive complexes. It seemed reasonable that an oligosaccharide of NAG of chain length greater than three containing a β-linked *p*-nitrophenyl group at its reducing end would function more efficiently as a substrate for lysozyme. Since, however, synthesis of such compounds is tedious, we made use of the glycosyl transferase activity of lysozyme which proceeds with retention of configuration (see preceding section) to provide these saccharides. On incubation of lysozyme (4×10^{-4} M) with chitotetraose (6×10^{-3} M) and *p*-nitrophenyl-β-NAG (2.5×10^{-2} M), release of *p*-nitrophenol was observed. After showing that the enzyme and *p*-nitrophenyl-β-NAG alone did not give rise to free nitrophenol and that neither did *p*-nitrophenyl-β-NAG and chitotetraose in the absence of enzyme, it was evident that the scheme outlined in Figure 2 was serving to gen-

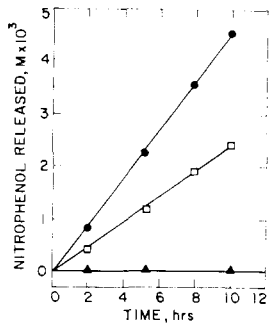

FIGURE 3: Time study of the lysozyme-mediated release of *p*-nitrophenol from mixtures of the enzyme (4×10^{-4} M) and chitotetraose (6×10^{-3} M) with (a) *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (5.0×10^{-2} M), —●—; (b) *p*-nitrophenyl β-D-glucoside (5.0×10^{-2} M), —□—; and (c) *p*-nitrophenyl α-D-glucoside (5.0×10^{-2} M), —▲—.

erate p-nitrophenyl glycosides. Proof that this indeed was the case is presented later in this paper (Figure 7). These glycosides were of sufficient chain length for the enzyme to effect fairly rapid release of free nitrophenol from them. Figure 3 shows the release with time of free nitrophenol in the incubation mixture. The rate of release of *p*-nitrophenol was linear up to 10 hr, when the experiment was discontinued. At this point the enzyme had hydrolyzed approximately eight turnover numbers of *p*-nitrophenyl glycoside. It was realized that this method of generation of chromophoric substrates for lysozyme made it feasible, in principle, to test a variety of compounds in the system.

p-Nitrophenyl β-D-glucoside (2.5×10^{-2} M) was next tested and it was found that in the presence of lysozyme (4×10^{-4} M) and chitotetraose (6×10^{-3} M), *p*-nitrophenol was released at an appreciable rate, whereas in the presence of lysozyme alone, no free nitrophenol was observed. Figure 3 shows the rate of release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucoside in this system as compared with *p*-nitrophenyl β-D-glucosaminide. The observed rate for the glucoside was approximately half that seen for *p*-nitrophenyl-β-NAG. Figure 4 shows the effect of *p*-nitrophenyl β-D-glucoside concentration on the rate of release of *p*-nitrophenol in the presence of lysozyme (4×10^{-4} M) and chitotetraose (6×10^{-3} M). The rate of hydrolysis increased with increasing concentration of *p*-nitrophenyl glucoside until approximately 2×10^{-1} M. This maximal rate probably

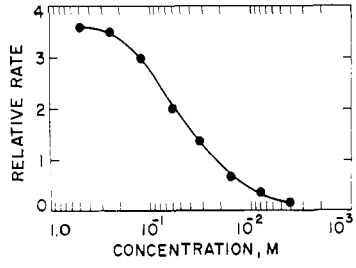

FIGURE 4: The effect of concentration of *p*-nitrophenyl β-D-glucoside on the rate of release of *p*-nitrophenol mediated by lysozyme (4×10^{-4} M) in the presence of chitotetraose (6×10^{-3} M).

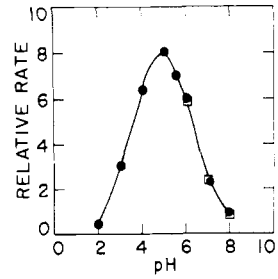

FIGURE 5: The pH profile of lysozyme- (4×10^{-4} M) catalyzed release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucoside (5×10^{-2} M) in the presence of chitotetraose (6×10^{-3} M).

reflects a complex situation in which increased rate of transfer to form *p*-nitrophenyl oligosaccharides suitable for hydrolysis may be opposed by increased inhibition of the enzyme due to binding of the monomer glycoside to the transferring site. The effect of pH on the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucoside in the presence of lysozyme and chitotetraose has also been measured and the results obtained are plotted in Figure 5. While it is difficult, because of the complexity of the reaction involved, to relate this curve to dissociations of side chains on the enzyme which could be involved in catalysis, the similarity of the pH dependence of the reaction being studied to the pH dependence of lysozyme-catalyzed hydrolysis of *p*-nitrophenyl-β-chitotriose (Osawa, 1966) is striking.

Other related *p*-nitrophenyl glycosides were tested for release of *p*-nitrophenol in the system described. No release of aglycone was observed with *p*-nitrophenyl β-D-galactoside, *p*-nitrophenyl β-D-xyloside, or *p*-nitrophenyl α-D-glucoside (Figure 3).

When, however, lysozyme (4×10^{-4} M) was incubated with chitotetraose (6×10^{-3} M) and *p*-nitrophenyl 2-deoxy-β-D-glucoside (2.0×10^{-2} M), release of *p*-nitrophenyl was observed as shown in Figure 6. The relative rate using this compound was sixteen times that observed using *p*-nitrophenyl β-D-glucoside. This result further demonstrates the variability of substituents which is permissible at C-2 of the pyranose ring which is undergoing glycosidic bond cleavage. This is in contrast to the stringent requirements already demonstrated for the orientations of substituents at C-1, C-4, and C-6 of the pyranose ring.

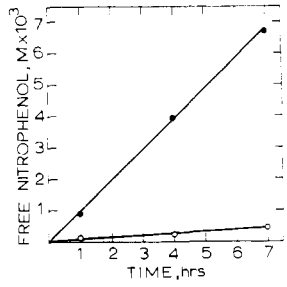

FIGURE 6: Time study of lysozyme-mediated release of *p*-nitrophenol from mixtures of the enzyme (4×10^{-4} M) and chitotetraose (6×10^{-3} M) with (a) *p*-nitrophenyl β-D-glucoside (2.5×10^{-2} M), —○—; and (b) *p*-nitrophenyl 2-deoxy-β-D-glucoside (2.5×10^{-2} M), —●—.

Demonstration of Transglycosylation to Acceptor Glycosides. Since *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, *p*-nitrophenyl β-D-glucoside, and *p*-nitrophenyl 2-deoxy-β-D-glucoside served as acceptors which lead to release of nitrophenol in the presence of lysozyme and chitotetraose while other related glycosides did not do so, it was of considerable interest to determine whether this failure was a result of an inability to generate oligomeric *p*-nitrophenyl glycosides by glycosyl transfer or of unsuitability of such oligomers once formed to serve as substrates for the enzyme. To this end we employed gel filtration on Bio-Gel P-2 columns to monitor the incubation mixtures of lysozyme, chitotetraose, and various *p*-nitrophenyl monosaccharides. Figure 7A–E shows the results obtained. It was evident that in all cases higher *p*-nitrophenyl oligosaccharides were formed. In those instances where nitrophenol was released a spectrum of such oligomers existed. In other instances where no release of nitrophenol was observed (using *p*-nitrophenyl β-D-galactoside and *p*-nitrophenyl α-D-glucoside), a high molecular weight *p*-nitrophenyl oligosaccharide fraction was formed but no appreciable amounts of lower homologs were obtained. The incubation mixture containing *p*-nitrophenyl β-D-xyloside appeared to be an exception, lower *p*-nitrophenol-containing oligosaccharides being formed without any evident release of *p*-nitrophenol. Thus it was evident that in all cases tested transglycosylation did occur but that certain stereochemical relationships must be necessary for catalysis to occur.

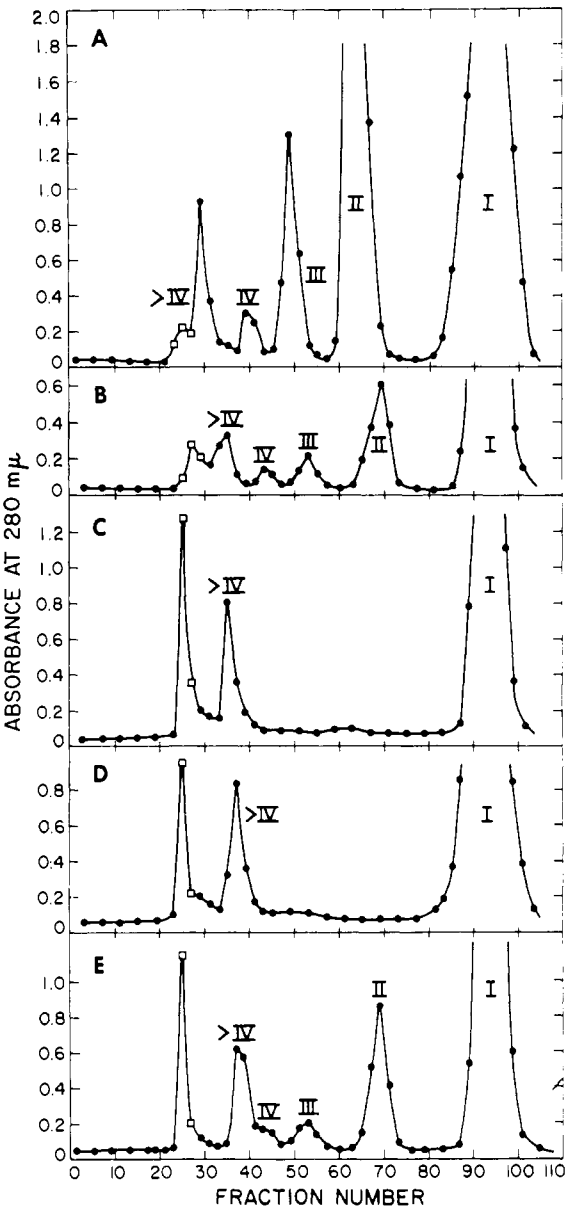

FIGURE 7: Gel filtration of Bio-Gel p-2 columns (1 × 100 cm) of aliquots of incubated mixtures of lysozyme (4×10^{-4} M) and chitotetraose (6×10^{-3} M) with (A) *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside; (B) *p*-nitrophenyl β-D-glucoside; (C) *p*-nitrophenyl α-D-glucoside; (D) *p*-nitrophenyl β-D-galactoside; and (E) *p*-nitrophenyl β-D) xyloside. Fractions containing protein (Foliu–Ciocaltiau) are shown as —□—; those containing *p*-nitrophenyl-containing components as —●—. Numerals I–IV denote *p*-nitrophenyl saccharides containing one to four or more sugar units, based on the known elution volume of *p*-nitrophenyl β-D-glucoside (fractions 86–100).

Discussion

Although it is clearly established that acid-catalyzed hydrolysis of glycosidic bonds proceeds by an SN1 mechanism (see Vernon, 1967; Cordes, 1967) and that base-catalyzed hydrolysis of certain aryl glycosides proceeds by SN2 mechanisms (Ballou, 1954; Gasman and Johnson, 1966), in no case has the mechanism of any enzyme-catalyzed cleavage of glycosidic bonds been elucidated. Obviously the studies on various model compounds may serve as an excellent guide to approach understanding such related enzymatic reactions.

On the basis of such studies the mechanisms portrayed in Figure 8 are considered possible for the lysozyme-catalyzed hydrolysis of glycosidic bonds. It has been shown (Rupley and Gates, 1967), that in the lysozyme-catalyzed hydrolysis of chitotriose, the bond cleaved is that between C_1 of a pyranoside ring and the glycosidic oxygen joining it to C_4 of an adjacent pyranose ring. All mechanistic pathways are depicted to occur on the surface of an enzyme molecule with a propinquous acidic and basic group (HA and B^-, respectively). All five pathways are depicted involve a general acid catalyzed step. Such a step, involving general acid catalysis, has been claimed (Capon, 1963) in model studies of the acid-catalyzed hydrolysis of *o*-carboxylphenyl β-D-glucoside, and of *o*-carboxyphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Piszkiewicz and Bruice, 1967), in another simple phenolic acetal (Capon and Smith, 1965) as well as in the hydrolysis of poly- and oligouronides (Smidsrød *et al.*, 1966). Mechanisms I, III, IV, and V can be written without this step, protonation occurring after the leaving group (RO^-) has departed.

Distinguishing between the Possible Mechanisms. Since it was shown that lysozyme-catalyzed transglycosylation of NAG from chitobiose to methanol results in formation of β-methyl-NAG without detectable amounts of α-methyl-NAG, it is clear that mechanism I (Figure 8) is not the pathway traversed by the substrate during

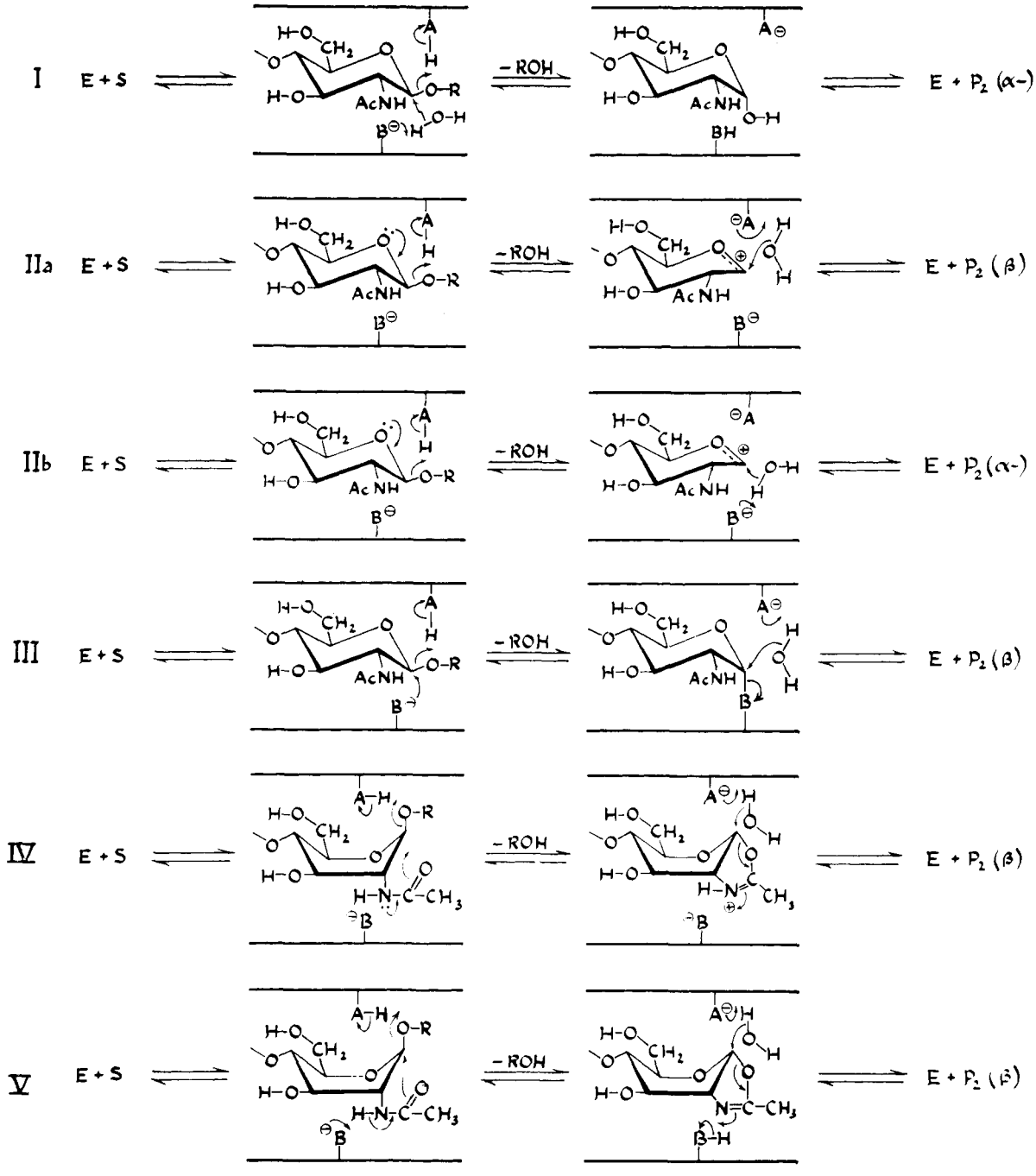

FIGURE 8: Depiction of mechanistic pathways possible for substrate during lysozyme-catalyzed cleavage of glycosidic bonds. AH and B denote an acid and a base, respectively, which are situated at the catalytic site of the enzyme. Further explanations are given in the text.

catalysis. The same result also eliminates mechanism IIb, *i.e.*, a carbonium ion giving rise to a product of inverted configuration. It does not, however, eliminate mechanism IIa, a carbonium ion intermediate giving rise to a product of retained configuration. In a separate study (Dahlquist *et al.*, 1968) of the transfer reaction, using ^{14}C-labeled chitobiose and quantitatively following the formation of methyl glycoside(s) of NAG by lysozyme in the presence of methanol, it was hoped to obtain positive evidence for mechanism IIa through finding a small percentage of α-methyl-NAG. It was shown, however, that the β-methyl product was obtained to an extent of at least 99.7%. The presence of small amounts of impurities did not allow quantitation beyond this value.

In the present study the transglycosylation reaction catalyzed by lysozyme has allowed the synthesis of a variety of new saccharides whose behavior in the presence of the enzyme have an important bearing on the mechanism. As expected, *p*-nitrophenyl oligosaccharides of NAG made in this way served as substrates. The finding that *p*-nitrophenyl oligosaccharides of the type nitrophenyl $(NAG)_n$-β-D-glucoside also served as substrates for lysozyme, *p*-nitrophenol being released through hy-

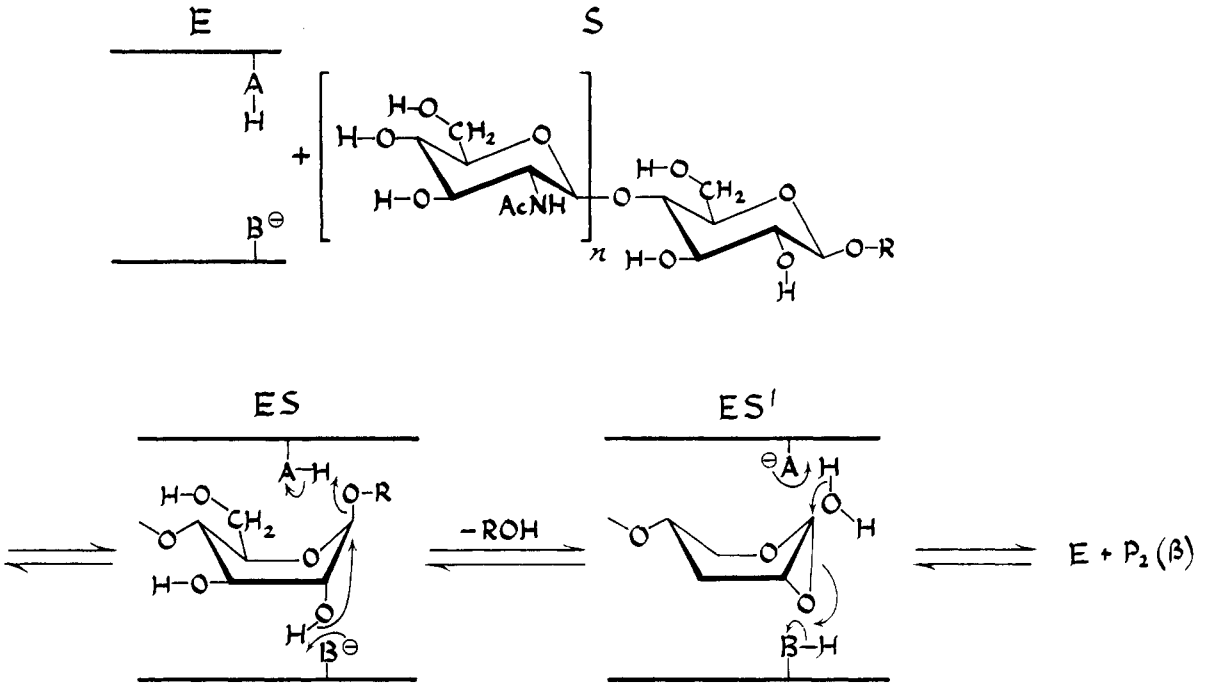

FIGURE 9: Possible mechanistic pathway for lysozyme-catalyzed hydrolysis of *p*-nitrophenyl β-D-glucopyranosides, involving general acid and general base catalysis by the enzyme and C-2 oxyanion participation by the substrate.

drolysis of the glucosidic bond, is of considerable interest. It had not previously been demonstrated that lysozyme could effect hydrolysis of glycosides other than those of oligosaccharides containing only NAG (β-[1–4] linked) or NAG-β-1–4-NAM units. Thus it is obvious that the acetamido groups of the natural substrates for the enzyme are necessary to effect binding, *i.e.*, formation of the enzyme–substrate complex, but are not *necessary* for catalysis to occur. In a separate study (T. Rand-Meir, F. W. Dahlquist, and M. A. Raftery, unpublished data) it has been shown that the apparent catalytic constant, k_{cat}, is almost the same for hydrolysis of *p*-nitrophenyl-NAG and *p*-nitrophenylglucosyl bonds by lysozyme. The present findings of the nonessentiality of an acetamido side chain for catalysis by the enzyme to occur eliminates the necessity of an anchimeric assistance, *i.e.*, mechanism IV, by substrate during catalysis by lysozyme. Such an anchimeric effect has been suggested for lysozyme-catalyzed hydrolysis of glycosidic bonds (Lowe, 1967a,b; Lowe *et al.*, 1967) and discussed at length by Piszkiewicz and Bruice (1967) as a possibility for the enzymic reaction.

Since the disposition of the 2-hydroxyl and the *p*-nitrophenyl glycosidic groups in the nitrophenyl β-glucosides is the same stereochemically as the 2-acetamido and p-nitrophenyl glycosidic groups in NAG-β-glycosides, the possibility of general base catalysis by the enzyme causing an anchimeric effect in the substrate (Figure 8V) was not eliminated by the finding that *p*-nitrophenyl β-glucosides served as substrates. It has recently been shown that, in the spontaneous hydrolysis of *o*- and *p*-nitrophenyl-NAG, the acetamido side-chain effects anchimeric assistance through formation of an oxazoline intermediate. Similarly, in the spontaneous hydrolysis of *o*- and *p*-nitrophenyl glucoside the 2-hydroxyl group was shown to also provide anchimeric assistance. In these studies (Piszkiewicz and Bruice, 1967) it was shown that participation by the acetamido side chain was more effective by a factor of 10^3 than participation by the 2-hydroxyl group. In the event, however, of the enzyme providing a general base for abstraction of a proton from the acetamido side chain as shown in mechanism V (Figure 8), the observed hydrolysis of *p*-nitrophenyl glucosidic bonds could be a result of general base catalysis by the enzyme through abstraction of a proton from the 2-hydroxyl of a glucose residue, thus allowing C-2 oxyanion participation as shown in Figure 9. Thus mechanism V could operate with substrates containing 2-acetamido groups. To test this possibility substances of the general formula *p*-nitrophenyl $(NAG)_n$-2-deoxy-β-D-glucoside were tested for lysozyme-catalyzed release of *p*-nitrophenol. As shown in Figure 6, nitrophenol was released from such compounds by the enzyme. Thus, mechanism V can be eliminated as an obligatory pathway on this basis. The greater rate exhibited by these substrates when compared with analogous glucose compounds (observed relative rates were 16:1, as shown in Figure 6) is of interest since it has been shown that acid-catalyzed hydrolysis of methyl 2-deoxy-β-D-glucoside proceeds at a rate approximately 10^3 times that of methyl β-D-glucoside (Overend *et al.*, 1962). However, it is not reasonable, due to the complexity of the enzyme-catalyzed reaction, to interpret the present results as indicating a carbonium ion mechanism.

The synthesis of various oligosaccharide glycosides, by means of the glycosyl transferase action of lysozyme, has therefore played a major role in distinguishing between several of the possible mechanistic pathways for substrates during hydrolysis by the enzyme. It is possible that the oligomeric *p*-nitrophenyl glycosides

formed in some instances where enzymatic release of *p*-nitrophenol was not found were not 1–4 linked. It is reasonable, however, to assume that they were β linked owing to the previously observed retention of configuration during transglycosylation. No attempt was made to determine whether or not 1–4 glycosidic bonds were present in these nonsubstrates. It has been shown (T. Rand-Meir, F. W. Dahlquist, and M. A. Raftery, unpublished data), that in those instances where *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl β-D-glucoside were used as transglycosylation acceptors, that β-1–4-glycosidic bonds were formed in the enzymatic transfer reaction. The results obtained using *p*-nitrophenyl β-D-xyloside as a transglycosylation acceptor deserve comment. It is evident from Figure 7E that transglycosylation occurred, yielding oligosaccharides containing *p*-nitrophenyl-β-D-xylose. However, no free nitrophenol was obtained, showing that such oligosaccharides did not serve as substrates. This failure could be the result of (a) inability to form β-1–4-linked glycosides during transglycosylation due to binding orientation of the acceptor p-nitrophenyl-β-D-xylose; (b) the necessity of having a 6-hydroxymethyl group for catalysis. It is considered unlikely that this group is directly involved in the catalytic mechanism due to unfavorable steric relationships in C-1, β-linked pyranosides. It is possible, however, as suggested by Blake *et al.* (1967b), that interaction with the enzyme of a 6-hydroxymethyl group of the pyranoside ring undergoing catalysis contributes energy to ring distortion and therefore to the mechanism.

From previous studies (Dahlquist *et al.*, 1966; Rupley *et al.*, 1967) it is quite clear that for good binding of saccharides to occur to the enzyme one or more (probably three) pyranose rings β-1–4-linked should possess 2-acetamido side chains. It has also been evident from the stability of lysozyme–saccharide complexes, *e.g.*, lysozyme–chitotriose complex (Phillips, 1967), that the catalytic site of the enzyme is outside the three contiguous strong binding sites for *N*-acetyl pyranoside rings. The present findings that *p*-nitrophenol is released from transfer products of chitotetraose and *p*-nitrophenyl β-D-glucoside constitute indirect evidence that the catalytic site may be removed by at least 6 Å from one end of the three strong binding sites for *N*-acetylamino sugars (no glucose derivative binds appreciably to the three strong binding sites (Rupley *et al.*, 1967)).

In conclusion it may be stated that of those reasonable mechanistic pathways (Figure 8) by means of which lysozyme could cleave glycosidic bonds the present studies have eliminated: (a) a single displacement mechanism; (b) a carbonium ion mechanism which gives rise to α-anomeric products; and have also shown that (c) a mechanism involving participation of the acetamido side chain of NAG-containing substrates and (d) a mechanism involving anchimeric assistance by the substrate due to general base catalysis by the enzyme are not necessary to explain catalysis. The remaining possibilities are: (a) a carbonium ion mechanism which gives rise stereospecifically to β-anomeric products; or (b) a double-displacement mechanism which also results in retention of configuration. This latter type of mechanism has been previously put forward as a scheme to account for retention of configuration (owing to two displacements at C_1) in enzymatic glycoside hydrolysis (Koshland, 1953). It should be emphasized that, although anichimeric assistance is not necessary to explain catalysis by lysozyme, the present findings do not of course exclude its occurrence in NAG substrates.

We cannot at the present time clearly distinguish between mechanisms IIa and III. If the carbonium ion intermediate exists it is possible that it is stabilized by a propinquous basic group since the lysozyme–intermediate complex is long lived, as evidenced by the transferase activity with numerous acceptors. It is possible that the base involved in stabilization of a carbonium ion as an ion pair (Winstein *et al.*, 1954, 1958) or in a covalently bonded glycosyl–enzyme intermediate is a carboxylate anion. The pH profiles for lysozyme-catalyzed hydrolysis of chitotriose (Osawa, 1966), chitopentaose (Rupley *et al.*, 1967), and *p*-nitrophenyl-β-D-glucosyl oligosaccharides in the present study indicate that a group of $pK_a = 3.5$–4.0 is involved in catalysis. These two mechanisms may be difficult to distinguish, since such distinction depends upon the distance of separation of the base and carbonium ion species. However, at present experiments designed to effect such distinction are being conducted.

References

Ballou, C. E. (1954), *Advan. Carbohydrate Chem. 9*, 59.

Blake, C. C. F. (1966), *New Sci.*, 333.

Blake, C. C. F., Johnson, Louise, N., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967b), *Proc. Roy. Soc. (London) B167*, 378.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1965), *Nature 206*, 757.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967a), *Proc. Roy. Soc. (London) B167*, 365.

Capon, B. (1963), *Tetrahedron Letters*, 911.

Capon, B., and Smith, M. C. (1965), *Chem. Commun. 7*, 523.

Chipman, D. M., Pollock, J. J., and Sharon, N. (1968), *J. Biol. Chem. 243*, 487.

Cordes, E. H. (1967), *Progr. Phys. Org. Chem. 4*, 1.

Dahlquist, F. W., Borders, C. L., Jacobson, G., and Raftery, M. A. (1968) *J. Biol. Chem.* (in press).

Dahlquist, F. W., Jao, L., and Raftery, M. A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 26.

Gasman, R. C., and Johnson, D. C. (1966), *J. Org. Chem. 31*, 1830.

Johnson, L. N., and Phillips, D. C. (1965), *Nature 206*, 761.

Koshland, D. E. (1953), *Biol. Rev. 28*, 416.

Lowe, G. (1967a), *Proc. Roy. Soc. (London) B167*, 431.

Lowe, G. (1967b), *Biochem. J. 104*, 893.

Lowe, G., Sheppard, G., Sinnott, N. L., and Williams, A. (1967), *Biochem. J. 104*, 893.

Lowry, O. H., Rosebrough, N. J., Farr, A., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mazur R. H., Ellis, B. W., Cammarta, P. S. (1962),

J. Biol. Chem. 237, 1619.
McIlvaine, T. C. (1921), *J. Biol. Chem. 49*, 183.
Osawa, T. (1966), *Carbohydrate Res. 1*, 435.
Osawa, T., and Nakasawa, Y. (1966), *Biochim. Biophys. Acta 130*, 56
Overend, W. G., Rees, C. W., and Sequeirz, J. S. (1962), *J. Chem. Soc., 3429*.
Phillips, D. C. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 484.
Piszkiewicz, D., and Bruice, T. C. (1967), *J. Am. Chem. Soc. 89*, 6237.
Raftery, M. A., Dahlquist, F. W., Borders, C. L., Jr., Jao, L., and Rand-Meir, T. (1968), *J. Biol. Chem.* (in press).
Rupley, J. A. (1964), *Biochim. Biophys. Acta 83*, 245.
Rupley, J. A., Butler, L. Gerring M., Hartdegan, F. J., and Pecoraro, R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1088.
Rupley, J. A., and Gates, V. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 496.
Schalegar, L. L., and Long, F. A. (1963), *Advan. Phys. Org. Chem. 1*, 1.
Smidsrød, O., Haug, A., and Larsen, B. (1966), *Acta Chem. Scand. 20*, 1026.
Vernon, C. A. (1967), *Proc. Roy. Soc. (London) B167*, 389.
Winstein, S., Clippinger, E., Fainberg, A. H., and Robinson, G. C. (1954), *Chem. Ind.*, 664.
Winstein, S., and Robinson, G. C. (1958), *J. Am. Chem. Soc. 80*, 169.
Zilliken, F., Rose, C. S., Braun, G. A., and György, P. (1957), *Arch. Biochem. Biophys. 54*, 392.

A Study of Muscle Polyribosomes and the Coprecipitation of Polyribosomes with Myosin*

Stuart M. Heywood,† Robert M. Dowben, and Alexander Rich

ABSTRACT: Polyribosomes from chick skeletal muscle were prepared and found to be very active in protein synthesis. The use of high salt concentrations (0.25 M) in the buffers is necessary because myosin coprecipitates with polysomes at low salt concentrations. The coprecipitation is not limited to a particular size class of polysomes. The addition of myosin to polysomes from tissues other than muscle also results in coprecipitation. This phenomenon can be utilized to prepare purified, enriched polysomes.

Muscle is a tissue uniquely suited for the study of the biosynthesis of relatively insoluble structural proteins and their organization into an architecturally complex pattern. Muscle also lends itself for studies of the effects of hormones on protein synthesis. A number of investigators have addressed themselves to this problem by attempting to prepare cell-free polyribosomal systems from skeletal muscle capable of incorporating labeled amino acids into trichloroacetic acid precipitable material (Breuer *et al.*, 1964; Earl and Korner, 1965; Rampersad *et al.*, 1965; Strohman, 1966). In an earlier report (Heywood *et al.*, 1967), we described a cell-free polyribosomal system prepared from chick embryo skeletal muscle which was very active in incorporating labeled amino acids into proteins, including material identified as myosin. In that report it was noted that skeletal muscle polysomes tended to coprecipitate with myosin. In this communication, we report further studies characterizing the myosin-synthesizing system and describe in detail the characteristics of the interaction between myosin and polysomes.

Experimental Procedure

Leg muscles from 14-day-old chick embryos dissected free of skin and bone were homogenized in an equal volume of ice-cold M buffer (0.25 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris, pH 7.4) by five strokes in a loose-fitting Dounce homogenizer (clearance 0.12 mm). More vigorous homogenization or the use of a more tightly fitting homogenizer resulted in degradation of polysomes. The homogenate obtained from about 0.7 g of muscle was centrifuged at 10,000*g* for 10 min to remove mitochondria and cell debris. The cytoplasmic supernatant was then layered on 27 ml of a 15–40% (w/w) linear sucrose gradient in M buffer. After centrifugation for 2 hr at 25,000 rpm in a Spinco No. 25.1 rotor at −18°, fractions were collected from the botton of the tube for absorbance measurements at 260 mμ and assays of radioactivity.

For some preparations, polyribosomes were isolated by coprecipitation with myosin at low salt concentration. The cytoplasmic extracts of muscle and other tissues prepared as outlined above were diluted by the

* From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received May 10, 1968.* Supported by grants from the U. S. Public Health Service, the National Science Foundation, and the National Foundation for Neuromuscular Diseases.

† U. S. Public Health Service postdoctoral fellow. Present address: Department of Biology, University of Connecticut, Storrs, Conn.