Studies on the Mechanism of Action of Uridine Diphosphate *N*-Acetylglucosamine 2-Epimerase*

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ABSTRACT: The uridine diphosphate *N*-acetylglucosamine 2-epimerase of rat liver cleaves uridine diphosphate *N*-acetylmannosamine to *N*-acetylmannosamine and uridine diphosphate; no trace of uridine diphosphate *N*-acetylglucosamine could be detected in the reaction mixture. When the reaction is carried out in tritiated water, the *N*-acetylmannosamine formed bears a label at C-2 but the activity thus introduced is lower than that in *N*-acetylmannosamine produced under identical conditions from uridine diphosphate *N*-acetylglucosamine. Uridine diphosphate *N*-acetylmannosamine recovered after brief exposure to the enzyme in tritiated water does not carry a label. On the basis of the available facts, it is suggested that uridine diphosphate *N*-acetylmannosamine is not an intermediate in the transformation of uridine diphosphate *N*-acetylglucosamine to *N*-acetylmannosamine catalyzed by 2-epimerase but is capable of serving as an alternative substrate. A mechanistic scheme which appears to be in harmony with the known facts is presented. Evidence indicating that the 2-epimerase reaction is catalyzed by a single enzyme has been obtained and attention is drawn to its possible role in the biogenesis of *N*-acetylmannosamine-containing macromolecules.

Uridine diphosphate *N*-acetylglucosamine 2-epimerase[1] catalyzes the conversion of UDPGlcNAc into ManNAc and UDP (Cardini and Leloir, 1957; Comb and Roseman, 1958). This transformation appears to be unique among those of nucleotide sugars inasmuch as the newly formed sugar is not nucleotide bound. The enzyme is specific for UDPGlcNAc, being inactive toward UDPGalNAc, UDPGlcNGlyc, GlcNAc-1-P, and GlcNAc (Cardini and Leloir, 1957; Spivak and Roseman, 1966; Hardesty, 1967, 1968). Unfortunately, efforts to purify this 2-epimerase and to study the mechanism of its action have been hampered by its extreme lability. However, Spivak and Roseman (1966) have found that the enzyme is stabilized to some degree by the presence of UDP or uridine and these authors were able to purify it 140-fold. During the course of their studies, they observed that the 2-epimerase has a pH optimum at 6.7 and another at 7.7; in addition, they observed an apparent lag in the formation of UDP which led them to conclude that the overall reaction might take place in two steps, namely, epimerization followed by hydrolysis, as in eq 1 and 2. However, evidence obtained earlier does not appear to support this mechanism. Glaser (1960) found that [2-^{3}H]ManNAc was formed from UDPGlcNAc when the reaction was carried out in tritiated water while no tritium was found in the UDPGlcNAc isolated from the incomplete reaction. These observations indicate that labeling must take place during the second step if the sequence above is accepted. It is difficult, however, to visualize a mechanism for the hydrolysis of the sugar moiety from the nucleotide in such a manner as to involve replacement of the proton at C-2.

$$\text{ULPGlcNAc} \rightleftarrows \text{UDPManNAc} \quad (1)$$

$$\text{UDPManNAc} + H_2O \rightleftarrows \text{UDP} + \text{ManNAc} \quad (2)$$

We wish now to report that we have in part confirmed the observations of Glaser (1960) and to describe a further study of the mechanism of the action of the 2-epimerase through the use of UDPManNAc as a substrate.

Materials and Methods

Chemicals. UDPManNAc was synthesized as described in the preceding paper (Salo and Fletcher, 1970) and UDPGlcNAc was purchased from Sigma Chemical Co. Calcium phosphate gel, streptomycin sulfate, Tris (heavy metal free, A grade), and EDTA were purchased from Calbiochem Corp. Tritiated water (25 mCi/g) was purchased from the New England Nuclear Corp. Uridine and UDP were obtained from P-L Biochemicals, Inc.

Analytical Methods. Paper chromatography was carried out on Whatman paper (No. 1 or No. 3MM) using either solvent A (pyridine–ethyl acetate–water, 2:5:7, upper phase, McFarren *et al.*, 1951) or solvent B (95% ethanol–1 M ammonium acetate, pH 3.8, 75:30, Paladini and Leloir, 1952). Paper electrophoresis, the detection of compounds on paper, and gas–liquid partition chromatography were carried out as described earlier (Salo and Fletcher, 1970). Arabinose was assayed by the phenol–sulfuric acid method (Hodge and Hofreiter, 1962) and arabinonic acid by the periodate–chromatropic acid procedure (Speck, 1962). Protein was estimated by the method of Lowry *et al.* (1951). Radioactivity was

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[1] The following abbreviations are used: UDP-*N*-acetylglucosamine 2-epimerase, 2-epimerase; UDP-*N*-acetylglucosamine, UDPGlcNAc; UDP-*N*-acetylmannosamine, UDPManNAc; UDP-*N*-acetylgalactosamine, UDPGalNAc, UDP-*N*-glycolylglucosamine, UDPGlcNGlyc; UDP-glucose, UDPGlc; *N*-acetylmannosamine, ManNAc; *N*-acetylglucosamine, GlcNAc; *N*-acetylglucosamine 1-phosphate, GlcNAc-1-P. All of the sugars are of the D series and in the pyranose form.

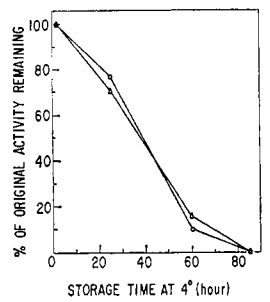

FIGURE 1: The loss of activity of 2-epimerase toward UDPGlcNAc (○—○) and UDPManNAc (△—△) on storage at 4°.

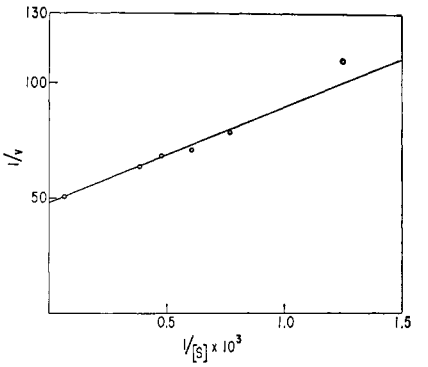

FIGURE 2: Lineweaver–Burk plot for UDPManNAc. $K_m = 9 \times 10^{-4}$ M.

estimated with a Packard Tri-Carb liquid scintillation spectrometer. Aqueous samples were counted in Bray solution (Bray, 1960).

Enzyme Preparation. UDPGlcNAc 2-epimerase was prepared and assayed using essentially the methods of Spivak and Roseman (1966). A mixture of fresh rat liver (25 g), buffer (0.005 M potassium phosphate, 50 ml), and glass beads (3 mm diameter, 50 ml) was homogenized for 1 min in a Waring blender connected to a Variac set at 30 V. The resulting slurry was filtered through one thickness of cheesecloth and then centrifuged at 35,000*g* for 15 min. Subsequent purification through step 4 was exactly as described by Spivak and Roseman (1966) except that nucleic acids were removed with streptomycin sulfate rather than with polymyxin sulfate. Preparations were used immediately after completion of step 4; they routinely had a specific activity of *ca.* 0.23 IU/mg of protein.

Results

Reactivity of UDPManNAc with 2-Epimerase. UDPManNAc (2 μM) was incubated at 37° for 40 min with 2-epimerase (0.02 IU) in a reaction mixture (0.9 ml) containing Tris buffer (200 μM, pH 7.5) and $MgSO_4$ (50 μM). The reaction was terminated by heating at 100° for 1.5 min and, after centrifuging off the denatured protein, a sample of the supernatant solution was applied to 3MM paper along with standard samples of UDP, UDPManNAc, and ManNAc. After development with solvent B, the chromatogram was examined for ultraviolet-absorbing compounds and for reducing sugars. The presence of UDP, unreacted UDPManNAc, and of an *N*-acetylhexosamine was detected. The remainder of the reaction mixture was chromatographed in preparative fashion on 3MM paper using solvent A and the *N*-acetylhexosamine band was eluted for rechromatography on No. 1 paper with solvent A; the material proved to be chromatographically indistinguishable from ManNAc. A control, made by boiling a solution of the enzyme, produced no ManNAc.

Ratio of Products. UDP to ManNAc. The reaction described above was repeated but scaled down to 1 μM of UDPManNAc. Two 0.05-ml aliquots were removed and applied to 3MM paper together with standard samples of UDP. After chromatography, using solvent B, each of the UDP spots was cut out and eluted with water. The eluates were adjusted to a volume of 5.0 ml and the UDP was determined (OD_{262}, ϵ 10^4 M^{-1}). An additional two 0.05-ml aliquots of the reaction mixture were passed over a small dual-bed column of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-). The effluent and washings were concentrated to dryness and then each was dissolved in a known volume of water and assayed for ManNAc. Estimated in this manner, the ratio of UDP to ManNAc averaged 0.98.

Loss of Activity of 2-Epimerase toward UDPGlcNAc and UDPManNAc. A fresh preparation of 2-epimerase was assayed for activity toward UDPGlcNAc and UDPManNAc. The enzyme was then stored at +4° and reassayed after 25, 60, and 85 hr; the results are portrayed in Figure 1.

K_m for UDPManNAc. The assay of Spivak and Roseman (1966) was used, varying the concentration of UDPManNAc in the presence of 0.004 IU of 2-epimerase. Figure 2 shows the data presented in the conventional Lineweaver–Burk plot. The function is linear and yields a K_m value of 9×10^{-4} M. Hardesty (1967, 1968) has reported a K_m for UDPGlcNAc of 5×10^{-4} M.

Reversal of Reaction, UDPManNAc to UDPGlcNAc. Paper chromatography systems available fail to separate UDPGlcNAc and UDPManNAc; in order to ascertain whether the action of 2-epimerase involves an equilibrium between these two substances (as depicted in eq 1), recourse was had to chromatography of the constituent *N*-acetylhexosamines which are readily separable in several chromatographic systems. The sugar nucleotide (UDPManNAc, 14.5 μM) was incubated with 2-epimerase (0.25 IU), Tris buffer (pH 7.5, 740 μM), and magnesium sulfate (185 μM) in a total volume of 3.1 ml. After 6 min at 37°, the reaction was stopped by heating at 100° for 1.5 min. The denatured protein was removed by centrifugation and the supernatant solution was lyophilized. The residue was dissolved in water (*ca.* 0.5 ml) and the solution was placed on a column (2.9 × 33 cm) of Sephadex G-10. Elution was carried out with distilled water, the eluate being collected in 5-ml fractions. Fractions 6–11 contained material absorbing at 262 nm (UDP and UDPhexoseNAc); they were pooled and lyophilized. The residue was dissolved in water (0.5 ml) and the solution was streaked on water-washed Whatman No. 3MM paper. After chromatography in solvent B, the UDPhexoseNAc band was eluted and the eluate was lyophilized to give a residue (8.2 μM) which was dissolved in 0.01 N hydrochloric acid (2 ml). The solution was heated at 100° for 20 min as was also a control consisting of ManNAc (8 μM) in 0.01 N hydrochloric acid; the control was carried through the subsequent

TABLE I: The Specific Activity of Tritiated ManNAc.[a]

Substrate	Sp Act. (cpm/μmole) Expt 1	Expt 2
UDPGlcNAc	9,670	11,940
UDPManNAc	8,448	10,340

[a] Each reaction mixture contained: UDPhexoseNAc (8 μM), 2-epimerase (0.57 IU), Tris buffer (pH 7.5, 200 μM), magnesium sulfate (50 μM), and tritiated water (0.32 ml, 25 mCi/g) in a total volume of 1.0 ml. The reactions were incubated at 37° for 1 hr.

work-up. The hydrolysate was cooled and passed over a small dual-bed column of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-) (0.5 × 3 cm of each). The effluent was concentrated to dryness *in vacuo* on a rotary evaporator and the residue was examined by paper chromatography using solvent A. A sample was converted into its trimethylsilyl derivative and chromatographed on a column of SE-52 (Salo and Fletcher, 1970). Neither system gave evidence for the presence of GlcNAc in either the UDPhexoseNAc or in the ManNAc control.

Reactions Carried out in Tritiated Water. UDPGlcNAc and UDPManNAc were separately incubated under identical conditions (detailed in Table I) with 2-epimerase in tritiated water. After heating to stop each reaction, the denatured protein was removed by centrifugation and the supernatant solution was lyophilized to give a residue which was dissolved in water (1 ml). The solution was passed over a dual-bed column of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-) (0.5 × 3 cm of each). The combined effluent and washings were concentrated to a volume of *ca.* 1 ml, streaked on water-washed 3MM paper, and chromatographed in solvent A. The ManNAc band was eluted and its specific activity was determined, the results being shown in Table I. The specific activity of tritiated ManNAc derived from UDPManNAc was 12.6 and 13.3% lower (expt 1 and 2) than the specific activity of the tritiated ManNAc derived from UDPGlcNAc. Rechromatography failed to alter the observed specific activities.

Since UDPManNAc yielded tritiated ManNAc, it was of interest to determine whether UDPManNAc recovered from an incomplete reaction was labeled. The UDPManNAc was therefore incubated with 2-epimerase as described in Table I, but the reaction was terminated after 10 min. The UDPManNAc (5 μM) was isolated as described earlier in this paper and counted; it was devoid of radioactivity.

Glaser (1960) noted that tritium was not incorporated into ManNAc when the sugar was used as a substrate in tritiated water. Incubation of ManNAc under the conditions described in Table I failed to result in incorporation of tritium and the presence of UDP (8 μM) was without effect.

Degradation of Tritium-Labeled ManNAc Derived from UDPManNAc. Glaser (1960) showed that the product of the action of 2-epimerase on UDPGlcNAc in tritiated water was [2-^{3}H]ManNAc. Using methods similar to those employed by Glaser, we investigated the position of the label in ManNAc derived from UDPManNAc. The tritiated ManNAc (5 μM) was N deacetylated by heating for 3 hr in 2 N hydrochloric acid in a sealed tube at 100°. The tube was cooled and the contents were evaporated to dryness on a rotary evaporator. Distilled water was evaporated three times *in vacuo* from the residue. The resulting mannosamine hydrochloride was purified by paper chromatography on water-washed Whatman No. 3MM paper using solvent A and then degraded to arabinose with ninhydrin (Stoffyn and Jeanloz, 1954). The arabinose was isolated by paper chromatography (solvent A, water-washed Whatman No. 3MM) and its specific activity was determined. It was then diluted to 500 cpm/μmole with unlabeled arabinose and oxidized to arabinonic acid (Schaffer and Isbell, 1963). The arabinonic acid was purified by paper electrophoresis and its specific activity was determined. The results of the degradation are given in Table II and clearly show that the tritiated ManNAc derived from UDPManNAc is 2-^{3}H.

TABLE II: The Degradation of Tritium-Labeled ManNAc Derived from UDPManNAc.

Compound	Sp Act. (cpm/μmole)
ManNAc	8448
Arabinose	8411
Arabinonic acid	0

Discussion

A mechanistic interpretation here must take the following items into account. (1) The failure to detect UDPManNAc in the transformation of UDPGlcNAc to ManNAc (Glaser, 1960). (2) The formation of [2-^{3}H]ManNAc from both UDPGlcNAc and UDPManNAc when these are acted upon by 2-epimerase in tritiated water. (3) The lower specific activity of the [2-^{3}H]ManNAc when derived from UDPManNAc than when formed from UDPGlcNAc. (4) The failure to detect UDPGlcNAc when the 2-epimerase acts on UDPManNAc. (5) The absence of tritium incorporation in UDPGlcNAc and UDPManNAc recovered after brief exposure to 2-epimerase in tritiated water.

At the outset, it seems clear that the mechanism shown in eq 1 is untenable. Items 4 and 5 argue against the reversible step 1 while item 3 is not consistent with the idea that labeling occurs in the second step. Let us then turn to an alternative hypothesis for the reaction catalyzed by 2-epimerase. Instead of serving as an intermediate in the transformation of UDPGlcNAc to ManNAc, UDPManNAc may simply be an alternate substrate for the system. A possible scheme is outlined in Figure 3 and is in harmony with the known facts.

It is reasonable to assume that formation of the glycosyl enzyme (GlcNAc) (reaction 1) is actually or virtually an irreversible reaction, thus preventing formation of a detectable amount of UDPGlcNAc from UDPManNAc and incorporation of tritium into substrates (item 5). The role of UDPManNAc as an alternate substrate for the system is represented by reaction 7; like reaction 1, this step must be regarded as irreversible in view of the failure of Glaser (1960) to detect

UDPManNAc in the transformation of UDPGlcNAc to ManNAc (item 1); item 5 also argues against the reversibility of reaction 7. It should be noted that the glycosyl enzyme (ManNAc), formed through reaction 7, may undergo subsequent transformation in either of two directions. On the one hand, it may participate in reactions 5, 4, and 3, incorporating tritium at C-2 *via* this route. On the other hand, the glycosyl enzyme (ManNAc) may hydrolyze directly (reaction 6) to ManNAc. These considerations account for items 2 and 3; the results of the labeling experiments require that 87% of the glycosyl enzyme (ManNAc) equilibrate through reactions 5, 4, and 3 while 13% is hydrolyzed directly. Thus the glycosyl enzyme (ManNAc) derived from UDPManNAc is only partly exposed to labeling while that derived from UDPGlcNAc must all go through reactions 3, 4, and 5 and is thus fully exposed to labeling.

The occurrence of keto intermediates in the transformations of nucleotide sugars is well documented (see reviews by Glaser, 1963, Leloir, 1964, and Neufeld and Ginsburg, 1965). Their intermediacy in the present reaction requires that the 2-epimerase contain or utilize a cofactor such as NAD^+ (or $NADP^+$). While we have observed no enhancement of activity upon addition of NAD^+ or $NADP^+$ it is not excluded that the enzyme may contain a tightly bound cofactor. The extreme lability of the enzyme has hampered purification and subsequent study of the possible cofactor content.

The significance of the double pH optima observed by Spivak and Roseman (1966) is not specifically clarified by the proposed scheme.

Since the 2-epimerase reaction was discovered, the question has remained whether it is catalyzed by one or two enzymes. The data in Figure 1, showing the loss of activity of the 2-epimerase toward UDPGlcNAc and UDPManNAc, appear to support the view that but one enzyme is involved.

While mannosamine has been reported as a constituent of bacterial polysaccharides (Shabarova *et al.*, 1962; Pickering, 1965; Lüderitz *et al.*, 1968) and an unconfirmed report suggests it as a component of a polysaccharide in the liver of the squid (Rosenberg and Zamenhof, 1962), it has not, as yet, been encountered in mammalian polysaccharides, glycolipids, or glycoproteins. While the glycosyl enzyme (ManNAc) in the suggested mechanism simply transfers ManNAc to water, it is possible that it could as well transfer ManNAc to other appropriate acceptors, thereby serving as an effective means of utilizing UDPGlcNAc as a donor of ManNAc in the synthesis of larger molecules containing this sugar moiety.

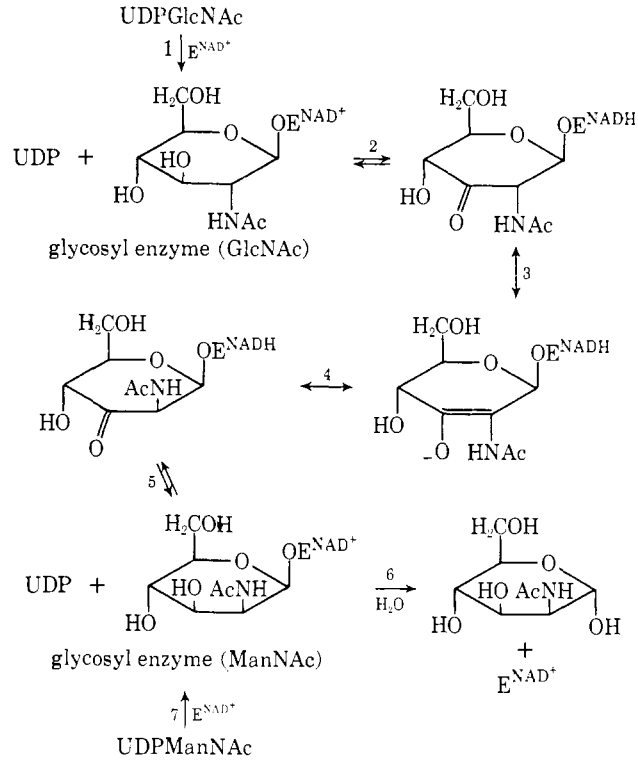

FIGURE 3: Possible pathway for the action of UDPGlcNAc 2-epimerase.

Acknowledgments

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