URIDINE DIPHOSPHOGALACTOSE 4-EPIMERASE

FROM BOVINE MAMMARY TISSUE

By

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CHAPTER I

INTRODUCTION

The enzyme uridine diphosphogalactose 4-epimerase (EC[1] 5.1.3.2) (UDPGal 4-epimerase) was first described by Leloir (1) in an extract from galactose-adapted yeast. UDPG was shown to be converted to UDPGal in a system known to convert Gal-1-P to G-1-P by a so-called galactoaldenase. After some confusion about naming, the enzyme which catalyzes the reaction UDPGal $\rightleftharpoons$ UDPG was named UDPGal 4-epimerase (2).

UDPGal 4-epimerase is believed to be essential for the direct interconversion of galactose and glucose and has been shown to be present in a number of sources from both the plant and animal kingdoms (3). It is important for the utilization of galactose as an energy source by way of the hexose phosphate pathway. When galactose is present as a nutrient, it is phosphorylated to form Gal-1-P. The interconversion of hexose phosphate then takes place according to the following reactions in which

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[1]The following abbreviations are used: EC, Enzyme Commission; UDPGal, uridine diphosphogalactose; UDPG, uridine diphosphoglucose; UDP, uridine diphosphate; UMP, uridine monophosphate; Gal-1-P, galactose-1-phosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; TDPGal, thymidine diphosphogalactose; TDPG, thymidine diphosphoglucose; NAD^+, nicotinamide-adenine dinucleotide; NADH, nicotinamide-adenine dinucleotide (reduced form); ATP, adenosine triphosphate; ADP, adenosine diphosphate; 6-PG, 6-phosphogluconate; UDPGA, uridine diphosphate glucuronic acid; UMP, uridine monophosphate; PCMB, p-chloromercuribenzoate; Tris, Tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; TEA, triethylamine; E. coli, Escherchia coli; S. fragilis, Saccharomyces fragilis.

UDPG functions catalytically in the Gal-1-P uridyl transferase reaction.

$$\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal}$$

$$\text{UDPGal} \rightleftharpoons \text{UDPG}$$

The over-all result of these reactions is the conversion of galactose to G-1-P which is readily used for energy.

UDPGal 4-epimerase is an important enzyme for the synthesis from glucose of the galactose moiety of complex biological materials such as galactolipids, lactose, cell wall components, and the blood group substances. Galactolipids are predominant in the myelinated substance of the nervous system of mammalian organisms. The UDPGal formed from glucose is the galactosyl donor in the reaction

$$\text{UDPGal} + \text{sphingosine} \longrightarrow \text{psychosine} + \text{UDP}$$

which is catalyzed by a brain enzyme (4).

UDPGal is also the galactosyl donor for lactose formation in mammary tissue.

$$\text{UDPGal} + \text{glucose} \longrightarrow \text{lactose} + \text{UDP}$$

Maxwell et al. (6) found that UDPGal 4-epimerase activity is increased two to three-fold during lactation in the rat and Shatton et al. (7) showed a 300-fold increase during lactation in rat mammary glands and in mammary tumors. The enzyme level drops sharply upon regression of the mammary tissue and reaches a level comparable to the pre-partum period. It is most probable that UDPGal 4-epimerase and the other enzymes responsible for lactose biosynthesis are under hormonal control. UDPGal 4-epimerase has not been purified from mammary tissue and it is the

purpose of this investigation to purify this enzyme from bovine mammary tissue and to study some of its chemical and physical properties.

CHAPTER II

LITERATURE REVIEW

Epimerase Reactions

Epimerization has been defined as the selective inversion of configuration at a single asymmetric center occurring in a compound containing more than one such center (3). This type of reaction is catalyzed in living systems by a group of enzymes called epimerases. According to the Enzyme Commission, epimerases are enzymes of the Isomerase class (5) and subclass (1) Racemases and Epimerases. These enzymes are of interest not only because of their important position in metabolic pathways, but also because of the mechanism by which the reaction proceeds.

Epimerization reactions have been found in a number of carbohydrate metabolic pathways, particularly among nucleotide sugar compounds. UDPGal 4-epimerase has been found in mammalian systems, yeast, bacteria and plants (3). Neufeld et al. (8) has demonstrated the enzyme UDP-L-arabinose 4-epimerase (EC 5.1.3.b) in mung bean seedling extracts which catalyzes the interconversion of UDP-L-arabinose and UDP-D-xylose. This enzyme was not separated from the UDPGal 4-epimerase present in the same extract. A particulate fraction from mung bean seedlings was found to contain UDP-D-galacturonic acid 4-epimerase (EC 5.1.3.c) which converts UDP-D-galacturonic acid into UDP-D-glucuronic acid (9). UDP-N-acetylglucosamine 4-epimerase (EC 5.1.3.d), which converts UDP-N-acetylglucosamine

into UDP-N-acetylgalactosamine, has been found in calf liver acetone powder (10), rat liver and Bacillus subtilis extracts (11). Jacobson and Davidson (12) have found a UDP-D-glucuronic acid 5-epimerase in rabbit skin which converts UDP-glucuronic acid to UDP-iduronic acid. An enzyme which catalyzes the conversion of TDPG to TDPGal has been shown to be present in bacteria (13, 14) and germinating mung beans (15).

Enzymes which catalyze the epimerization of sugar phosphates are important in pentose metabolism. D-Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1), the enzyme catalyzing the interconversion of D-ribulose-5-phosphate and D-xylulose-5-phosphate has been found in mammalian tissue, plants and bacteria (3). An enzyme which catalyzes the interconversion of the L-isomers of these two pentose phosphates has been shown to be present in extracts of Aerobacter aerogenes (16). L-Ribulose-5-phosphate 4-epimerase (EC 5.1.3.a), an enzyme found only in bacteria, catalyzes the interconversion of L-ribulose-5-phosphate and D-xylulose-5-phosphate.

Enzymes have been found which catalyze epimerization about carbon atoms bound to functional groups other than hydroxyl groups. An enzyme system in rat liver (17) catalyzes the formation of free N-acetyl-D-mannosamine from UDP-N-acetylglucosamine, a reaction in which the configuration at a carbon atom linked to an N-acetylamine group is changed. Ghosh and Roseman (18, 19) have purified two enzymes which epimerizes the N-acylamine group of two N-acylhexosamines. N-Acyl-D-glucosamine-6-phosphate 2-epimerase, which catalyzes the interconversion of N-acyl-D-glucosamine-6-phosphate and N-acyl-D-mannosamine-6-phosphate, was purified from bacterial extracts. N-Acyl-D-glucosamine 2-epimerase was purified from hog kidney and catalyzes the interconversion of N-acyl-D-glucosamine and N-acyl-D-mannosamine. Hydroxyproline 2-epimerase (EC 5.1.1.a), which

catalyzes an inversion of configuration involving the carboxyl groups at carbon 2 of hydroxylproline, has been purified from Pseudomonas striata (20).

Isolation of UDPGal 4-Epimerase from Various Sources

UDPGal 4-epimerase has been found in a wide variety of sources including yeast (21), bacteria (22), mammary, liver and brain tissue (6), and mung bean seedlings (8). Maxwell (24) has obtained the enzyme with the highest reported specific activity (406 units/mg protein) from calf liver. Highly purified preparations have been also obtained from Saccharomyces fragilis (25) and Escherchia coli (23, 26). The enzyme has not been crystallized or purified as a homogeneous protein from any source.

Comparison of the Properties of UDPGal 4-Epimerase from Different Sources

UDPGal 4-epimerase purified from different sources has many similar properties. The epimerase isolated from calf liver (24) has a Km for UDPGal of 5×10^{-5} M and 9×10^{-5} M for UDPG. At equilibrium the ratio of UDPG:UDPGal is 3:1. The enzyme has a pH optimum range of 8.0 to 9.6. The calf liver enzyme lost 50 percent of its activity when stored two weeks at -20° in 0.25 M glycylglycine buffer, pH 7.5, but lost no activity when stored lyophilized at -20°.

The enzyme purified from E. coli (23) has a Km for UDPGal of 1.6×10^{-4} M and has a UDPG:UDPGal ratio of 3.5:1 at equilibrium. The pH optimum range was between pH 8.0 and 8.5. The molecular weight was $7.9 (\pm 0.8) \times 10^{4}$ as determined by velocity and equilibrium sedimentation measurements.

Imae et al. (26), who also purified UDPGal 4-epimerase from E. coli, found the pH optimum to be between 7.5 and 9.0. The Km was 1.6 X 10^{-4} M for UDPGal and 1.0 X 10^{-3} M for UDPG and the UDPG:UDPGal ratio at equilibrium was 3.5:1.

The epimerase purified from S. fragilis (25) had a broad pH optimum between 8.0 and 9.5 which closely corresponds with the calf liver and E. coli enzyme. At equilibrium, the ratio of UDPG to UDPGal was 3:1. This value is approximately the same as that found for calf liver, E. coli, and in S. fragilis (1) by Leloir.

Darrow and Creveling (27) found that cations increase the activity of the epimerase purified 200 fold from Candida pseudotropicalis. Polyvalent amines such as spermine and spermidine were shown to stabilize the enzyme at low substrate concentrations and to increase the maximal reaction velocity at high substrate concentrations.

Requirement for NAD^+

NAD^+ has been shown to be a cofactor for UDPGal 4-epimerase isolated from liver, bacteria and yeast. The calf liver epimerase requires the addition of exogenous NAD^+ to be fully active and it is inhibited by NADH (24). Half-maximal velocity is reached with the purified enzyme at 2 X 10^{-7} M NAD^+; and when an equal amount of NADH is added, the reaction is about 70 percent inhibited. This inhibition can be partially overcome by increasing the NAD^+ concentration. Crude preparations of the enzyme are stimulated by NAD^+, and NAD^+ becomes increasingly stimulating with purification.

Wilson and Hogness (23) showed that NAD^+ was tightly bound to the enzyme purified from E. coli. Using the methyl ethyl ketone procedure

for determining N-substituted nicotinamide derivatives (28), they were able to find one NAD^+ residue per enzyme molecule. The absorption spectrum of the purified enzyme had no peak in the 300 to 400 mμ range, indicating that the bound dinucleotide was in the oxidized form. The difference spectrum between the enzyme with substrate and without substrate was very similar to the spectrum of NADH, with the peak shifted from 340 to 345 mμ.

NAD^+ was also shown to be tightly bound to the enzyme purified from S. fragilis (25). Maxwell et al. (21) found that it has a fluorescence spectrum which seems to correspond to that of NADH, both with respect to the spectrum and to the efficiency of the exciting light as a function of wave length (29). The enzyme has an activation maximum at 350 mμ and an emission maximum at 450 mμ. It was shown with the purified enzyme (25) that the fluorescence, NAD^+ content, and epimerase activity off a DEAE cellulose column were very closely correlated. These results suggest that the fluorescence behavior is a characteristic of the enzyme-NAD^+ complex. Other pyridine nucleotide-enzyme complexes have been shown to fluoresce only when the pyridine nucleotide is in the reduced form (30). However, NAD^+ can form fluorescent complexes with cyanide and a number of small organic molecules (31). Thus the significance of the relationship between fluorescence and bound NAD^+ is not fully understood.

Velick (32) has shown that protein-bound NAD^+ in crystalline muscle triosephosphate dehydrogenase (EC 4.3.2.1) can be removed by p-chloromercuribenzoate. When yeast and liver epimerase were treated with PCMB, they lost activity, but the E. coli (26) enzyme was unaffected. The activity of the yeast enzyme could not be restored by adding cysteine and NAD^+. However, when the PCMB-treated enzyme was precipitated with

ammonium sulfate and redissolved, its activity could be partially restored by the addition of NAD^+ and cysteine, but not by cysteine alone (25). Calf liver epimerase is 90 percent inhibited by 10^{-5} M PCMB and the inhibition is completely overcome by 7.6×10^{-3} M cysteine. This shows the inhibition is not due to the removal of NAD^+.

Other epimerase enzymes have been tested for NAD^+ requirement and varying results have been obtained. Both UDP-glucuronic acid 5-epimerase (12) and UDP-N-acetylglucosamine 4-epimerase (33) require catalytic amounts of NAD^+ and are also inhibited by NADH. Several methods for NAD^+ detection were used in determining if L-ribulose-5-phosphate 4-epimerase (34, 35) and D-ribulose-5-phosphate 3-epimerase (35, 36) had bound NAD^+ but the results were negative. No attempt to find NAD^+ associated with other epimerase enzymes have been reported.

Table I summarizes the properties of various epimerase enzymes. Table II is a summary of the properties of UDPGal 4-epimerase isolated from various source material.

Proposed Mechanisms of the UDPGal 4-Epimerase Reaction

A number of suggestions have been made for the mechanisms by which the epimerization reaction proceeds (40). They include: (I) ring closure to an inositol followed by ring opening at a different position, (II) cleavage to 3 carbon or other fragments followed by recondensation of the fragments, (III) removal of water between carbon atoms 3 and 4 or between carbon atoms 4 and 5 followed by rehydration, (IV) dehydrogenation between carbon atoms 3 and 4, or 4 and 5, to give an ene-diol intermediate, followed by rehydrogenation, (V) nucleophilic attack by OH^- of the medium to bring about inversion of configuration, and (VI) oxidation of the

TABLE I

A SUMMARY OF EPIMERASE REACTIONS

Enzyme	Sources	Reaction Catalyzed	Km	pH Optimum	NAD^+ Required
D-Ribulose-5-phosphate 3-epimerase	*Lactobacillus pentosus* (36) mammalian, plants, bacteria	D-Xylulose-5-P $\rightleftharpoons$ D-Ribulose-5-P	Xu-5-P, 5.0×10^{-4} M Ru-5-P, 1.0×10^{-3}	7-8.5	No
L-Ribulose-5-phosphate 4-epimerase	*Aerobacter aerogenes* (37)	L-Ribulose-5-P $\rightleftharpoons$ D-Xylulose-5-P	Rib-5-P, 1×10^{-4} M	8.5-9.5	
	L. plantarum (34)		Rib-5-P, 1×10^{-3} M	7-9	---
UDP-Glucuronic acid 4-epimerase	*Streptococcus pneumoniea* (38)	UDP-Glucuronic acid $\rightleftharpoons$ UDP-Galacturonic acid	---	---	---
	Mung bean seedlings (9)				
UDP-L-arabinose 4-epimerase	Mung bean seedlings (8)	UDP-L-arabinose $\rightleftharpoons$ UDP-D-xylose	---	---	---
UDP-N-acetylglucosamine 4-epimerase	Calf liver (10)	UDP-N-acetylglucosamine $\rightleftharpoons$ UDP-N-acetylgalactosamine	---	8.8	yes
	Bacillus subtilis (11)				
TDP-Acetylylglucosamine 4-epimerase	*Pseudomonas aeruginous* (39)	TDP-N-acetylglucosamine $\rightleftharpoons$ TDP-N-acetylgalactosamine	---	---	---
UDP-D-glucuronic acid 5-epimerase	Rabbit skin (33)	UDP-D-glucuronic acid $\rightleftharpoons$ UDP-L-iduronic acid	---	7.9 (Tris) 9.0 (Glycine)	Yes
Hydroxyproline 3-epimerase	*Pseudomonas striata* (20)	Hydroxyproline (D & L) $\rightleftharpoons$ Allohydroxyproline (D & L)	2.2×10^{-2} M	7.4-8.0	No
N-Acyl-D-glucosamine-6-P 2-epimerase	*Aerobacter cloacae* (18)	N-Acetyl-D-glucosamine-6-P $\rightleftharpoons$ N-Acetyl-D-mannosamine-6-P	---	---	No
N-Acyl-D-glucosamine 2-epimerase	Hog kidney (19)	N-Acetyl-D-glucosamine $\rightleftharpoons$ N-Acetyl-D-mannosamine[1]	---	---	No
---	Rat liver (17)	UDP-Acetylglucosamine $\rightleftharpoons$ N-Acetyl mannosamine	---	---	---

[1]Catalytic amounts of ATP required.

TABLE II

COMPARISON OF THE PROPERTIES OF UDPGAL 4-EPIMERASE FROM VARIOUS SOURCES

Source	Specific Activity $\left(\frac{\mu mole/min}{mg}\right)$	Km	NAD^+	Optimum pH
E. coli (23)	217	UDPGal, 1.6×10^{-4} M	Enzyme-bound	
E. coli (26)	114	UDPGal, 1.6×10^{-4} M UDPG, 1.0×10^{-3} M	Enzyme-bound	7.5-9.0
S. fragilis (25)	302	----	Enzyme-bound	8-9.5
Calf liver (24)	406	UDPGal, 5×10^{-5} M UDPG, 9×10^{-5} M NAD^+, 2×10^{-7} M	Needed in catalytic amounts[1]	8-9.6
Candida pseudotropicalis[2] (27)	(200-fold purification)	UDPGal, 1.1×10^{-4} M	----	----

[1]Enzyme is inhibited by NADH

[2]Enzyme is activated by spermine, spermidine, $MgCl_2$, NaCl, and several other monovalent and divalent cations.

secondary alcohol group at carbon 4 to a keto group, followed by reduction to give the other epimer.

Mechanisms I and II were shown to be inoperative by feeding rats C-1 and C-2 labeled galactose-^{14}C, isolating the glucose formed, and locating the position of the label in the glucose (41, 42). Since the label remained in its original position in both cases, it was concluded that inositol was not an intermediate and that the carbon chain was not ruptured.

Several experiments have been carried out with radioisotopes which eliminate mechanisms III, IV, and V as the operative one. It would be expected that hydrogen or oxygen from the medium would be involved in each of these mechanisms. Anderson et al. (43) used a bacterial extract to convert Gal-1-P to G-6-P in an $H_2{}^{18}O$ medium and found that no ^{18}O was incorporated into the glucose during the epimerization. Kowalsky and Koshland (44) did the same experiment in $T_2{}^{18}O$ medium and found no isotopes in the isolated glucose. Kalckar and Maxwell (2) used their purified calf liver epimerase to convert UDPGal to UDPG in 96 percent D_2O. They found that there was no change in reaction rate as would be expected if the deuterium was involved in the reaction. It was also shown that no tritium from the T_2O in the medium was incorporated into UDPG when the liver or yeast (25) enzyme was used.

McDonough and Wood (35) used the same type of isotope-incorporation experiments to study the mechanism of pentose phosphate epimerases from yeast. D-Ribulose-5-phosphate 3-epimerase incorporated one atom of tritium into ribulose-5-phosphate from T_2O in the medium. This would indicate a mechanism different from that of UDPGal 4-epimerase. Neither tritium nor ^{18}O was incorporated from T_2O or $H_2{}^{18}O$ by L-ribulose-5-

phosphate 4-epimerase, which indicates that its mechanism is similar to that of UDPGal 4-epimerase.

The oxidation-reduction mechanism (VI) appears to be the operative mechanism, not because of direct evidence, but because all the other proposed mechanisms have been probably eliminated. Wilson and Hogness (23) have postulated the following model:

$$S_1 + E\text{-}NAD^+ \rightleftharpoons E\text{-}NAD^+, S_1 \rightleftharpoons E\text{-}NADH, S_{ox} \rightleftharpoons E\text{-}NAD^+, S_2 \rightleftharpoons E\text{-}NAD^+ + S_2$$

where $E\text{-}NAD^+$ is the purified enzyme with bound NAD^+, S_1 is UDPG, S_2 is UDPGal, and S_{ox} is a hypothetical derivative of either UDP-hexose oxidized at carbon 4 of the hexose residue.

Several attempts to obtain direct evidence for the reaction mechanism have failed. Attempts to trap an intermediate in the reaction were unsuccessful (24). When the reaction was run in the presence of the carbonyl reagents thiosemicarbazide, hydroxylamine, or hydrazine, no derivative of a 4-keto intermediate was obtained. No NADH accumulated when the reaction was run in the presence of trapping agents. Attempts to accumulate an oxidized intermediate by coupling the reaction with NADH oxidase or with acetaldehyde and alcohol dehydrogenase were also unsuccessful.

Using the purified yeast and calf liver enzymes Maxwell (24, 25) tried to show incorporation into the sugar molecule of tritium from NAD^+ and NADH tritiated in the para position. It was assumed that the failure to observe incorporation of tritium resulted from the fact that the NAD^+ involved in the reaction was firmly bound to the enzyme, thus preventing the exogenous NAD^+ from being involved in the reaction.

Kohn *et al.* (40) gave rats galactose uniformly labeled with ^{14}C and at C-4 with tritium, isolated glucose and checked its radioactivity.

They found that the galactose administered and the glucose isolated both had the same $^{3}H:^{14}C$ ratio. This indicated that the same NAD^+ molecules are utilized for both the oxidation and reduction steps because of binding to the enzyme. The carbon-bound hydrogen removed to form a 4-keto intermediate would be replaced in the opposite steric configuration.

Bevill et al. (45, 46) have synthesized UDPG and UDPGal labeled with tritium in the 4-position and studied the isotope effect associated with the epimerase reaction. When k_T/k_H was measured for the reaction proceeding in both directions, values were found ranging from 3.0 to 1.5, depending on the method used in its calculation. Comparing these results with normal isotope effects (47) indicated that the 4-hydrogen is removed in a non-rate-determining step. Because the k_T/k_H ratio is greater than one, a reaction mechanism involving cleavage of a C-O or a C-C bond at carbon-4 does not fit the observed data. A mechanism consistent with all observations involves a transfer of the hydrogen at carbon-4 to the enzyme, followed immediately by the rate-determining step, which could be the rearrangement of the enzyme-substrate complex to allow the return of the hydrogen in the opposite configuration.

The larger value (3.0) obtained for the isotope effect could be explained by transfer of the 4-hydrogen to a nitrogen, and the lower value (1.5) by a transfer to carbon or oxygen. Transfer to sulfur would be definitely eliminated by these values. Even the lower value is somewhat high for transfer to NAD^+, but it is possible that enzyme binding might influence the values obtained for the isotope effect. They concluded that their data did not prove a direct hydrogen transfer to NAD^+, but did not preclude such a mechanism.

de Robichon-Szulmajster (48) has postulated a theoretical mechanism

based on two hydrogen acceptors, the uracil portion of the UDP hexose and NAD^+. UDPGal and UDPG have a structure such that uracil could be in position to accept one hydrogen from the hexose while the NAD^+ would accept the other hydrogen.

Stroud and Hassid (49) synthesized the 4-keto derivative of galacturonic acid-1-phosphate and arabinose-1-phosphate to try as intermediates in epimerase reactions. But when they tried to convert them to the UDP compounds with the mung bean pyrophosphorylase system, they could get no UDP-4-keto-galacturonic acid and UDP-4-keto-arabinose.

The mechanism by which the enzymatic epimerization takes place has not been definitely proved. Most investigators in this area would favor the hypothesis that the 4-keto hexose is an intermediate and that NAD^+ is the H^+ acceptor and donor involved.

CHAPTER III

METHODS AND MATERIALS

Materials

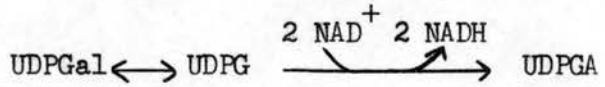
UDPG dehydrogenase (1.1.1.22) was purified from calf liver through step 5 of the procedure by Strominger (50). UDPG, NAD^+, PCMB, protamine sulfate and ATP were obtained from Sigma Chemical Co.; DEAE cellulose and Bio Gel P-10 from Calbiochem. Corp.; Sephadex G-100 from Pharmacia Fine Chemicals, Inc.; Dowex-1 from Dow Chemical Co.; Biodryex from Lövdalens Industri Aktiebolag, Centralpalatset, Stockholm C, Sweden; and activated charcoal from Barnebey-Cheney. All other chemicals used were of reagent quality.

Methods

Enzymatic Assays

UDPGal 4-epimerase was assayed by two methods. Assay I was a spectrophotometric method adapted from the method described by Maxwell (24) and was used for routine assays. Assay II was a two-step method used primarily in studies concerning NAD^+ and Km determinations.

In Assay I, the epimerase activity was measured by measuring UDPG formation by means of coupling the reaction to UDPG dehydrogenase. The increase in absorbance at 340 mμ was recorded on the Cary Model 14 spectrophotometer.



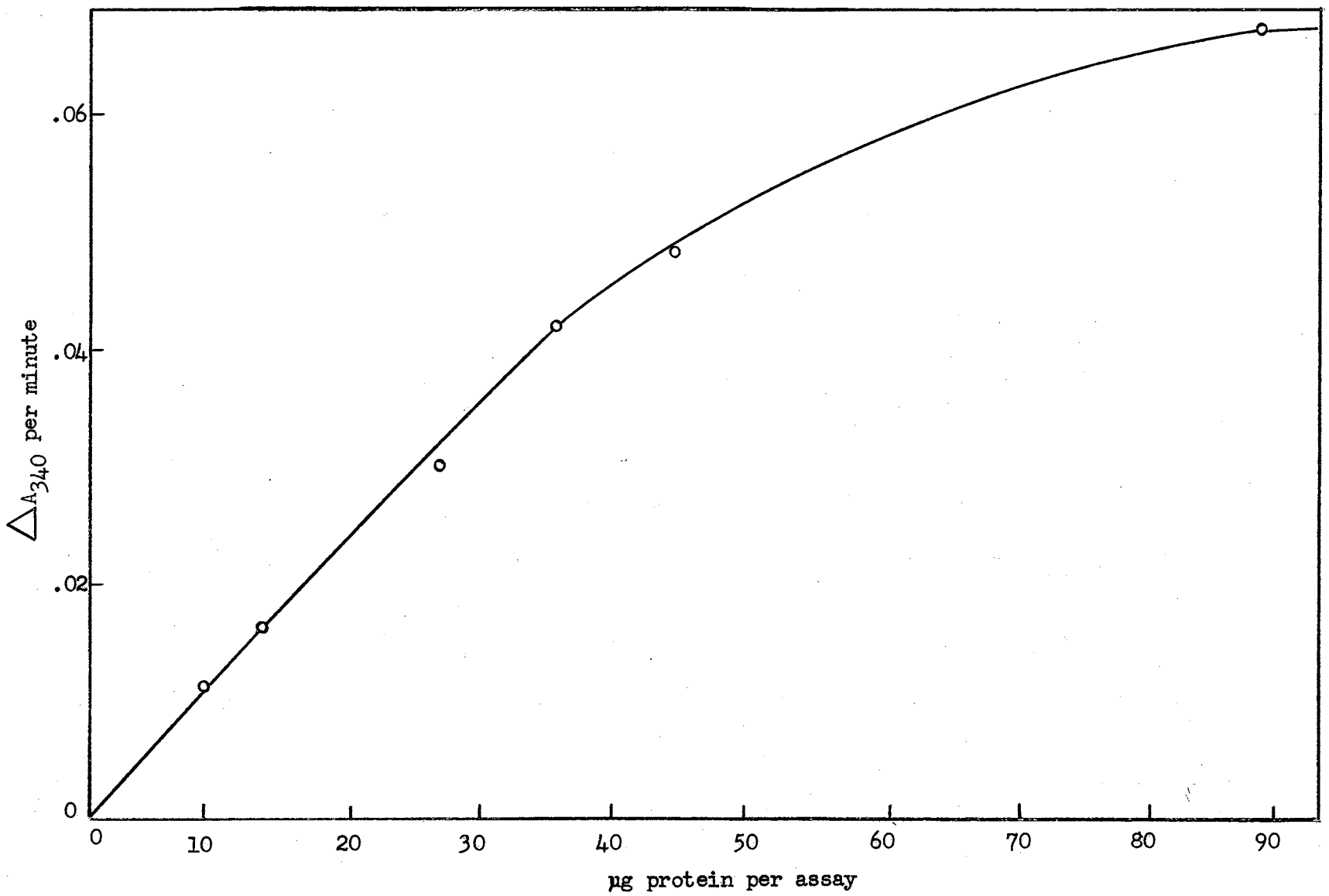
The reaction was carried out in a 0.75 ml cuvette containing 0.25 ml of 0.2 M glycine buffer, pH 8.7 (50 µmoles), 0.5 µmoles NAD^+, about 0.04 µmoles of UDPGal, sufficient UDPG dehydrogenase so that it was not rate-limiting, and sufficient enzyme and deionized water to give a final volume of 0.5 ml. The change in absorbance at 340 mµ was measured against a blank which contained all the above components except UDPGal. The rate of reaction was found to be proportional to enzyme concentration up to a change in absorbance of 0.04 per minute as shown in Figure 1. A unit of enzyme is defined as the amount that catalyzes the conversion of one µmole UDPGal to UDPG per minute.

Assay II was adapted from the procedure of Imae et al. (26). The enzyme was first incubated for ten minutes at 25° with approximately 0.08 µmoles UDPGal in 0.02 ml of 0.5 M glycine buffer, pH 8.7 (ten µmoles) and enough deionized water to give a final volume of 0.1 ml. The reaction was stopped by placing in boiling water for 90 seconds and then the reaction mixture was immediately put into an ice-bath. The reaction mixture was diluted to 0.5 ml with cold deionized water. The UDPG formed was measured with UDPG dehydrogenase in a reaction mixture which contained 0.5 ml of 0.2 M glycine buffer, pH 8.7 (100 µmoles), an excess of UDPG dehydrogenase (0.05 ml), one µmole NAD^+, 0.3 ml of the above incubation mixture, and sufficient deionized water to give a final volume of one ml. The total change in absorbance at 340 mµ was measured on the Cary Model 14 spectrophotometer, and the UDPG formed was calculated from the amount of NAD^+ reduced. The linearity of the assay for UDPG is shown in Figure 2 and the proportionality with respect to epimerase activity

Figure 1

Linearity of Assay I with Respect to Protein Concentration

Assay I for UDPGal 4-epimerase was conducted with varying amounts of the epimerase purified through Step III. The reaction mixture contained 50 μmoles glycine buffer, pH 8.7; 0.5 μmoles NAD^+, about 0.04 μmoles UDPGal; sufficient UDPGal 4-epimerase and deionized water to give a final volume of 0.5 ml in a 0.75 ml cuvette. The initial rate was measured on a Cary Model 14 spectrophotometer.


.06
.04
.02
0
$\triangle A_{340}$ per minute
0
10
20
30
40
50
60
70
80
90
µg protein per assay

is shown in Figure 3.

Other Methods

DEAE cellulose was prepared by washing with 0.1 N NaOH, 0.1 N HCl, 0.1 N NaOH, and then with a one M solution of the buffer to be used. After the acid and base washes, the cellulose was washed with distilled water until its pH was close to seven. The pH of the cellulose suspension in buffer was measured to insure that the pH of the cellulose was at the desired value. Observations had been made that the pH of the supernatent solution from a suspension of DEAE could differ from the DEAE cellulose suspension. Just before use, the cellulose was washed with the appropriate buffer until equilibrated. Equilibration was achieved by checking that the influent and effluent buffer solution had the same conductivity and pH. Such precautions are especially necessary when DEAE is equilibrated with low molarity buffers, e.g. 10-20 mM.

Protein concentrations were measured by the procedure of Lowry et al. (51). Fractions off columns were collected on a Buchler fraction collector. Conductivity measurements were made with a Radiometer conductivity meter.

Figure 2

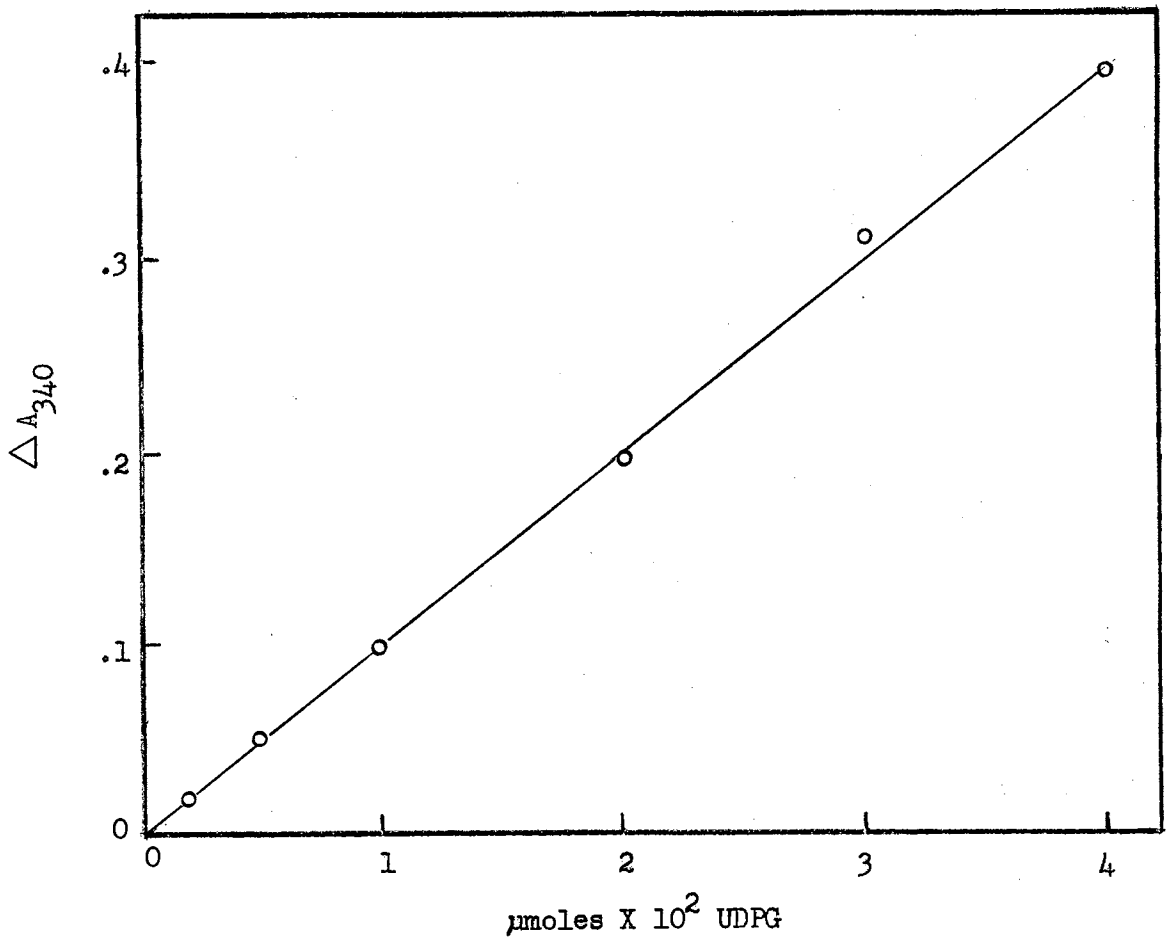
Quantitative Determination of UDPG by Assay II

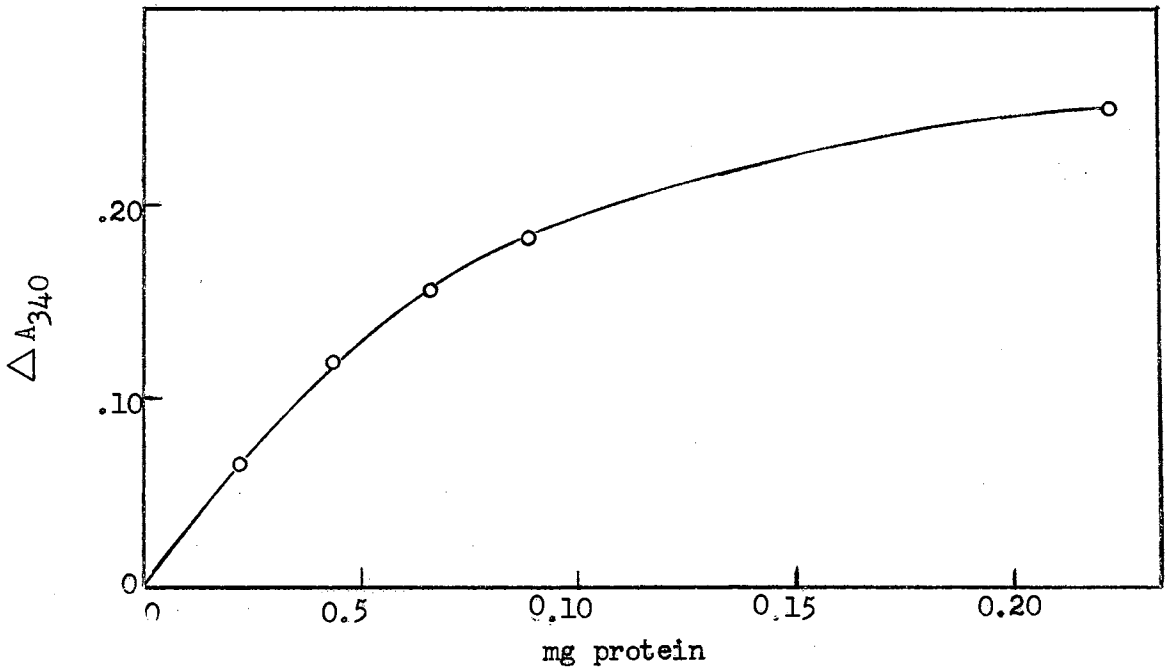
The total change in A_{340} was measured for a reaction mixture containing 100 μmoles glycine buffer, pH 8.7; one μmole NAD^+; an excess of UDPG dehydrogenase; and varying amounts of UDPG and deionized water to give a total volume of 1.0 ml. The change in absorbance at 340 mμ was measured with a Cary Model 14 spectrophotometer.

Figure 3

Linearity of Assay II with Respect to Protein Concentration

Assay II for UDPGal 4-epimerase was run using varying amounts of the epimerase purified through Step VI. The enzyme was first incubated with 0.08 μmoles of UDPGal and ten μmoles glycine buffer, pH 8.7, for ten minutes in a total volume of 0.1 ml. The reaction was stopped by boiling and diluted to 0.5 ml with deionized water. The UDPG formed was calculated from the total A_{340} change in a reaction mixture containing 100 μmole glycine buffer, pH 8.7; an excess of UDPG dehydrogenase; one μmole NAD^+; and an aliquot of the above reaction mixture in a final volume of 1.0 ml.


.4
.3
.2
.1
0
ΔA_{340}
0
1
2
3
4
μmoles X 10^2 UDPG


.20
.10
0
ΔA_{340}
0
0.5
0.10
0.15
0.20
mg protein

CHAPTER IV

UDPGALACTOSE SYNTHESIS

Three different methods were used in attempts to synthesize sufficient quantities of UDPGal. They were: (a) the enzymatic method described by Anderson et al. (52), (b) the chemical method of Okazhi et al. (53), and (c) the chemical method of Moffatt and Khorana (54, 55). Of these methods, only the third was successful in our laboratory.

Enzymatic Method

An enzyme system was isolated from S. fragilis which contained galactokinase, phosphoglucomutase and Gal-1-P uridyl transferase (52). With this system UDPGal could be synthesized from galactose by the following reactions:

$$\text{Gal} + \text{ATP} \longrightarrow \text{Gal-1-P} + \text{ADP}$$

$$\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{UDPGal} + \text{G-1-P}$$

$$\text{G-1-P} \rightleftharpoons \text{G-6-P} \longrightarrow \text{6-PG}$$

$$\text{UDPG} \longrightarrow \text{UDPGA}$$

After the enzymatic reaction was completed, the remaining UDPG was converted to UDPGA by the addition of purified UDPG dehydrogenase. After terminating the reaction by heating at 80° for two minutes, the solution was diluted with deionized water so that the conductivity was less than that of ten mM TEA-HCO_3 buffer.

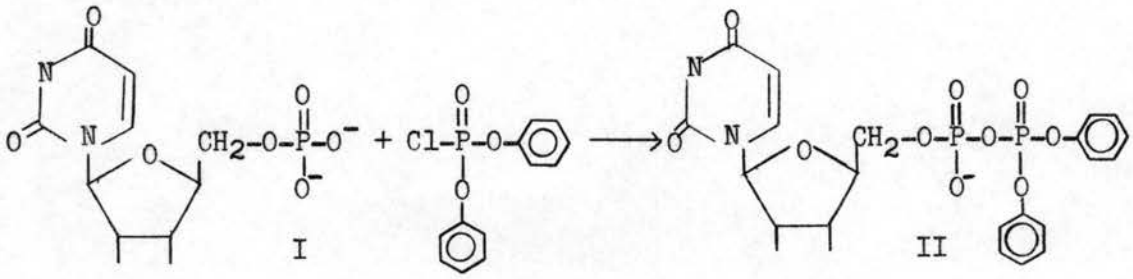
The nucleotide solution was put on a DEAE cellulose-HCO_3 column

which had been washed in deionized water and was eluted by a linear gradient of 0 to 0.4 M TEA-HCO_3 buffer, pH 7.4, using a total of 400 ml. Fractions found to contain nucleotide peaks (A_{260}) were pooled and lyophilized. The nucleotides were identified on paper or thin layer chromatography. A solvent system containing four volumes of one M ammonium acetate, pH 7.5, to 7.5 volumes of 95 percent ethanol was used for paper (Whatman No. 1) and MN 300 cellulose (Brinkmann) (56) thin layer chromatograms. DEAE cellulose (Brinkmann) thin layer chromatograms were run using 0.03 N HCL as a solvent (57).

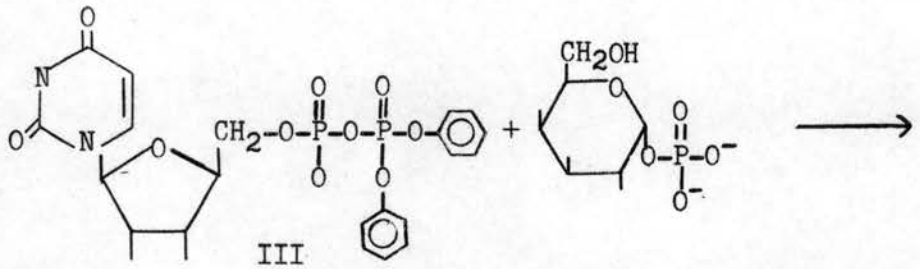
The enzymatic method failed to yield sufficient quantities of purified UDPGal as problems were encountered in keeping all enzymes active in the yeast preparation. When UDPGal was formed in the reaction mixture, it could not be separated completely from the other nucleotides on the DEAE cellulose column. Thus, it appeared that the method was not satisfactory to produce sufficient quantities of UDPGal. Hence efforts were directed toward chemical methods.

Michelson Synthesis

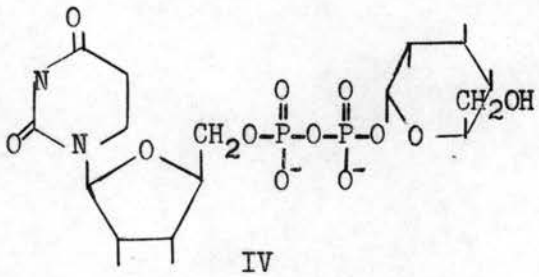
Attempts were made to synthesize UDPGal by adapting the method used for the synthesis of TDPG as described by Okazahi et al. (53). UDPGal (IV) is formed by the displacement of diphenyl phosphate from P^1-uridine 5'-P^2-diphenyl pyrophosphate (II) by ∝-D-galactose-1-phosphate (III), as shown in the scheme below.



I II



III

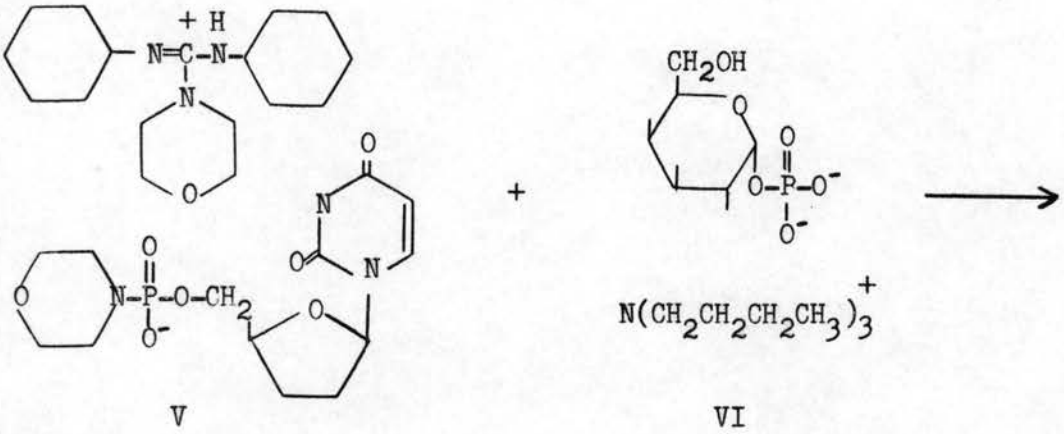


IV

α-D-Gal-1-P was prepared by the method of MacDonald (58) by heating B-D-galactose pentaacetate with crystalline phosphoric acid under five mm vacuum. When the tri-N-octylammonium salt of UMP was prepared, it would not dissolve in dioxane and hence, reacted poorly with diphenyl phosphorochloridate. Benzene and dimethylformamide were also tried as solvents but did not dissolve the tri-N-octylammonium salt. At this point the synthesis was discontinued. However, in a later paper Michelson (59) used a mixture of dioxane and dimethylformamide as the solvent for the tri-N-octylammonium salt of UMP.

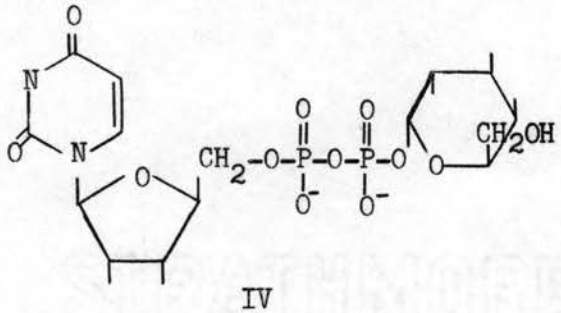
Moffatt and Khorana Synthesis

UDPGal was prepared by the method of Moffatt and Khorana et al. (54, 55). The 4-morpholine N, N'-dicyclohexylcarboxamidine salt of uridine-5' phosphoromorpholidate (V) was reacted with the tri-N-butylammonium salt (used instead of the tri-N-octylammonium salt) of Gal-1-P (VI).



V

VI



IV

The amine salt of Gal-1-P was prepared in a variety of ways and the following was found to be the most suitable. An aqueous solution of one mmole Gal-1-P in 25 ml water was passed through a 1.5 X 20 cm column of Dowex 50 (H^+ form) in the 4° cold room. The eluent was collected in a flask containing five ml water and one ml pyridine while stirring. This solution was condensed to about five ml on a rotory evaporator under vacuum. Two mmoles of redistilled tri-N-butylamine in four ml of pyridine were added very slowly with stirring until the solution became clear. This solution was brought to dryness on a rotory evaporator under vacuum and then dissolved in pyridine and redried three times. This preparation was also difficult to dissolve in pyridine and thus reduced yields. No problems were encountered with dissolving the morpholidate

in pyridine. Recently Verheyden et al. (60) have emphasized that the preparation of amine salts of phosphate esters require complete solution prior to the addition of the amine.

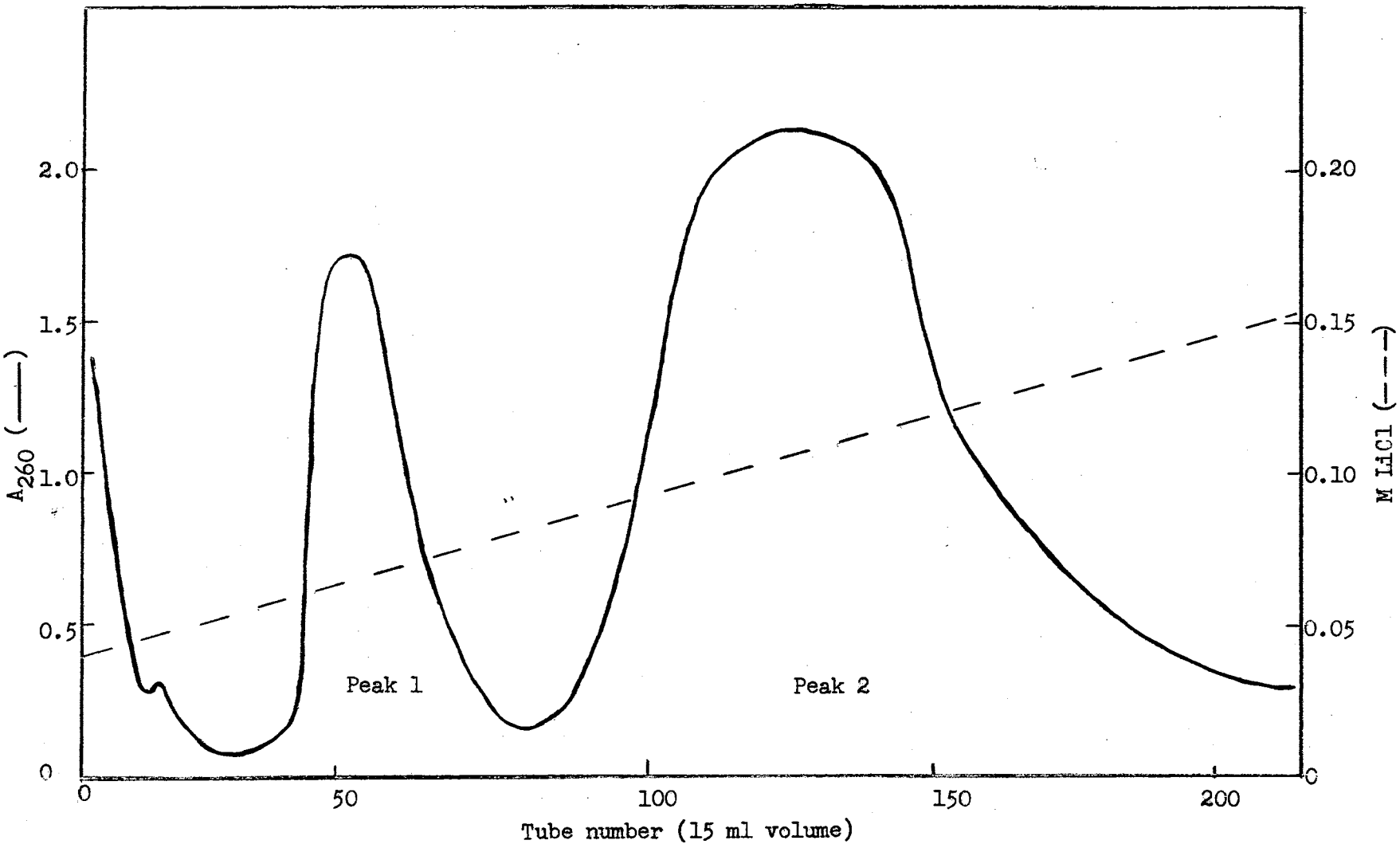
UDPGal was separated from the reaction mixture by eluting from a Dowex 1 (Cl^-) column with a linear gradient from 0.04 M LiCl, 0.003 N HCl; to 0.16 M LiCl, 0.003 N HCl using one l of each. An elution pattern is shown in Figure 4. The UDPGal was eluted at about 0.09 M LiCl.

This method yielded between 80 and 100 mg of the lithium salt of UDPGal from one mmole of the Gal-1-P salt and 0.5 mmole of the UMP-morpholidate. Thin layer chromatography of this preparation on cellulose (57) showed it was contaminated with a small amount of UMP. This preparation of UDPGal was used in the routine assays of UDPGal 4-epimerase. Experiments showed that UMP and UDPGal could not be separated on DEAE-HCO_3 with TEA-HCO_3, pH 7.5 as the eluting agent (0 to 0.3 M linear gradient). Consequently, UDPGal was repurified on the Dowex 1 (Cl^-) column as described above.

Figure 4

Purification of UDPGal on Dowex 1 (Cl^-)

UDPGal was purified by putting the reaction mixture on a 2.5 X 30 cm Dowex 1 (Cl^-) column and eluting with a linear gradient formed from one 1 each of 0.04 M LiCl, 0.003 N HCl and 0.16 M LiCl, 0.003 N HCl (---). Fractions of 15 ml were collected and monitored (A_{260}) on a Beckman DU spectrophotometer. The UDPGal (peak 2) was eluted off at about 0.09 M LiCl. Peak 1 was identified as UMP.


2.0
1.5
1.0
0.5
0
A_{260} (——)
0.20
0.15
0.10
0.05
0
M LiCl (– – –)
Peak 1
Peak 2
0
50
100
150
200
Tube number (15 ml volume)

CHAPTER V

ISOLATION OF UDPGAL 4-EPIMERASE FROM BOVINE MAMMARY TISSUE

Acetone Powder

An acetone powder of bovine mammary tissue was used as the starting material for the purification of UDPGal 4-epimerase since this avoids the difficulties encountered when enzymes are extracted directly from mammary tissue. That is, bovine mammary tissue contains large amounts of fat and connective tissue. The epimerase activity present in the acetone powder stored at -20° was found to be stable for several months whereas it was very unstable in mammary tissue extracts (61). Mammary glands were obtained from lactating grade cows at Wilson Packing Plant in Oklahoma City, and from lactating dairy cows which were kindly furnished by Dr. B. L. Larson, University of Illinois.

The tissue was obtained immediately after slaughter, cut into small pieces and frozen in powdered dry-ice. The frozen tissue was thawed to 0° and ground in a heavy-duty mechanical meat-grinder. The ground tissue, in 150 g portions, was extracted with ten volumes of acetone at -10° by blending in a large Waring blender for three periods of 20, 30, and 30 seconds. The temperature was maintained at about -10° by the addition of powdered dry-ice. The blended material was then filtered through shark skin filter paper under suction until almost dry while keeping the temperature at -10°. The moist cake was immediately

blended again for three 20-second periods in ten volumes of acetone at -10° as previously described. The blended material was then dried as much as possible on the suction filter funnel. The cake was air-dried by rubbing it with the hands and with a spatula on brown paper, and when dry it was sieved through a wire screen (No. 20). The powder was kept overnight at 4° in a desiccator with paraffin in order to absorb traces of acetone and then was stored at -20°.

Purification Steps

All steps were carried out in an ice-bath at 0° or in a cold room at 4° unless otherwise stated. Centrifugations were done in a Servall RC-2 at 16,000 X g for 30 minutes at 0°. Steps of the purification of UDPGal 4-epimerase are summarized in Table III.

Step I. Extraction. Twenty grams of the mammary tissue acetone powder were extracted with a composite buffer which contained 0.2 M Tris, ten mM MgCl, mM ethylenedinitrotetraacetic acid and mM mercaptoethanol, pH 7.4. After stirring for one hour, the suspension was centrifuged and then strained through glass wool to remove fatty material which collects on top of the supernatant solution.

Step II. Protamine Sulfate Fractionation. A two percent aqueous protamine sulfate solution (w/v) was added over a 15 minute period, with constant stirring, to the Step I supernatant. After the addition of one ml of protamine sulfate per 20 ml extract, stirring was continued for 20 minutes. The suspension was centrifuged and the precipitate was discarded.

Step III. Ammonium Sulfate Fractionation. The Step II supernatant was brought to 35 percent saturation by adding 209 g ammonium sulfate per

TABLE III

PURIFICATION OF UDPGAL 4-EPIMERASE

	ml	Total Units X 10^{-3}	Total mg Protein	Specific Activity (Units/mg Protein)
Step I Extraction	262	121.5	4870	24.9
Step II Protamine Sulfate Supernatant	260	105	4220	24.8
Step III Ammonium Sulfate Precipitate	46	92.8	2000	46.4
Step IV Calcium Phosphate Gel Supernatant	76	50.4	625	80.7
Step V After DEAE Cellulose Chromatography	12.5	43.2	105	411
Step VI After Sephadex G-100 Chromatography	5	15.7	29.2	537

liter of solution. The percent saturation was calculated from the table in reference (62) which is based on percent saturation at 23°. The solid ammonium sulfate was added over a 15 minute period and then the solution was allowed to stir 15 minutes in an ice-bath. The cloudy suspension was centrifuged and the precipitate was discarded. The 35 percent saturated solution was brought to 65 percent saturation by adding 200 g/l ammonium sulfate over a period of 15 minutes, allowed to stir for an additional 15 minutes and then centrifuged. The supernatant was discarded and the precipitate was dissolved in 30 to 35 ml of a buffer containing 0.1 M potassium phosphate, two mM mercaptoethanol, pH 7.6. The epimerase could be stored at this step at -20° for several weeks without substantial loss of activity.

Step IV. Calcium Phosphate Gel. The enzyme solution from Step III was desalted by passing through a 2.5 X 37 cm column of Bio Gel P-10 equilibrated in 25 mM potassium phosphate buffer, two mM mercaptoethanol, pH 7.6. This procedure increased the total volume of enzyme solution by one-third. The protein concentration was estimated from the 260:280 mμ absorbance ratio (63). A suspension of calcium phosphate gel (33.4 mg/ml, aged two years) was added to the enzyme solution over a period of 15 minutes to give a final concentration of one mg gel per mg protein. After stirring for 20 minutes, the gel was removed by centrifugation and was discarded. The majority of the epimerase activity remained in the supernatant solution but when the gel was washed with 0.1 M potassium phosphate buffer, pH 7.6, a protein solution of very low specific activity was obtained.

Step V. DEAE Column Chromatography. A column (2.5 X 15 cm) was packed with DEAE cellulose which had been equilibrated with 25 mM potassium

phosphate buffer, two mM mercaptoethanol, pH 7.6, as has been previously described. The enzymatic solution was put on the column and washed with 125 ml of the same buffer. A linear gradient was used to elute the enzyme using 500 ml each of the above buffer and 0.1 M potassium phosphate buffer, two mM mercaptoethanol, pH 7.6 (Figure 5). Fractions containing the enzyme peak were pooled and the enzyme was concentrated by precipitating with ammonium sulfate at 65 percent saturation while the pH was kept at 7.6 by the addition of two N ammonium hydroxide. The precipitate was dissolved in a small volume (about ten ml) of 0.1 M potassium phosphate, two mM mercaptoethanol, pH 7.6.

Step VI. Sephadex G-100 Chromatography. A 1 X 100 cm Sephadex G-100 column was equilibrated with 25 mM potassium phosphate buffer, two mM mercaptoethanol, pH 7.6, for three days. The enzyme from Step V was concentrated to one or two ml by placing it in a dialysis bag and covering it with Biodryex until the volume was sufficiently reduced. One ml of the concentrated enzyme was placed on the Sephadex column and was eluted with the same buffer used for equilibration. Fractions of two ml were collected and the fractions containing epimerase activity were pooled (Figure 6). The enzyme was concentrated by precipitating with ammonium sulfate at 65 percent saturation. This preparation could be stored at -20° for several weeks without significant loss of activity and was used for further studies of the enzyme. Purification of from 20 to 40 fold was obtained with the above procedure, depending on the source of mammary tissue.

Figure 5

DEAE Cellulose Chromatography of UDPGal 4-Epimerase

The enzyme solution was put on a 2.5 X 15 cm column of DEAE cellulose equilibrated with 25 mM potassium phosphate buffer, two mM mercaptoethanol, pH 7.6. After washing with 125 ml of the same buffer, a linear gradient was run, using 500 ml each of the above buffer and 0.1 M potassium phosphate, two mM mercaptoethanol, pH 7.6. Fractions of approximately seven ml were collected. Protein was monitored by A_{280} with a Beckman DU spectrophotometer (——). UDPGal 4-epimerase activity was followed by Assay I (·····).

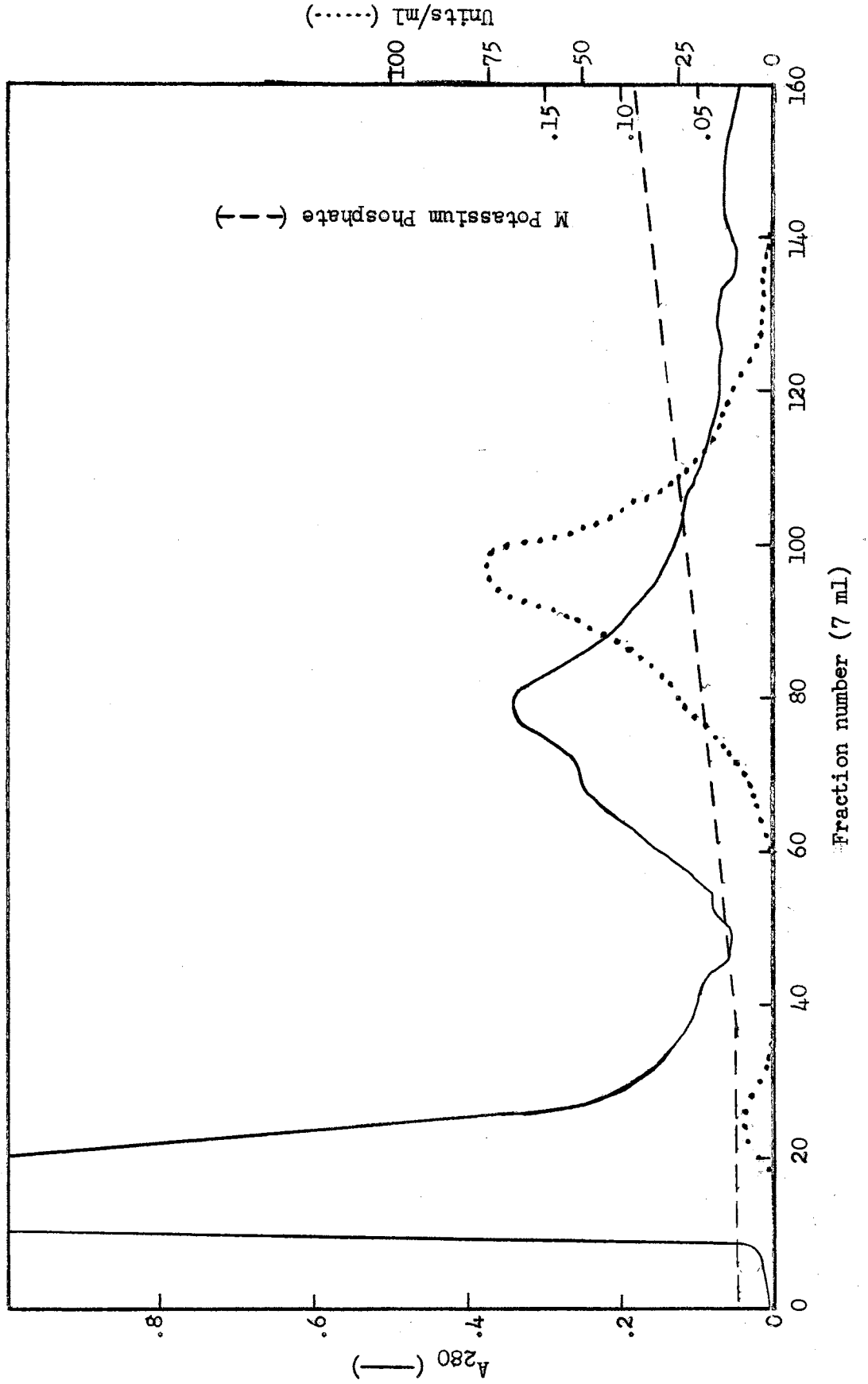
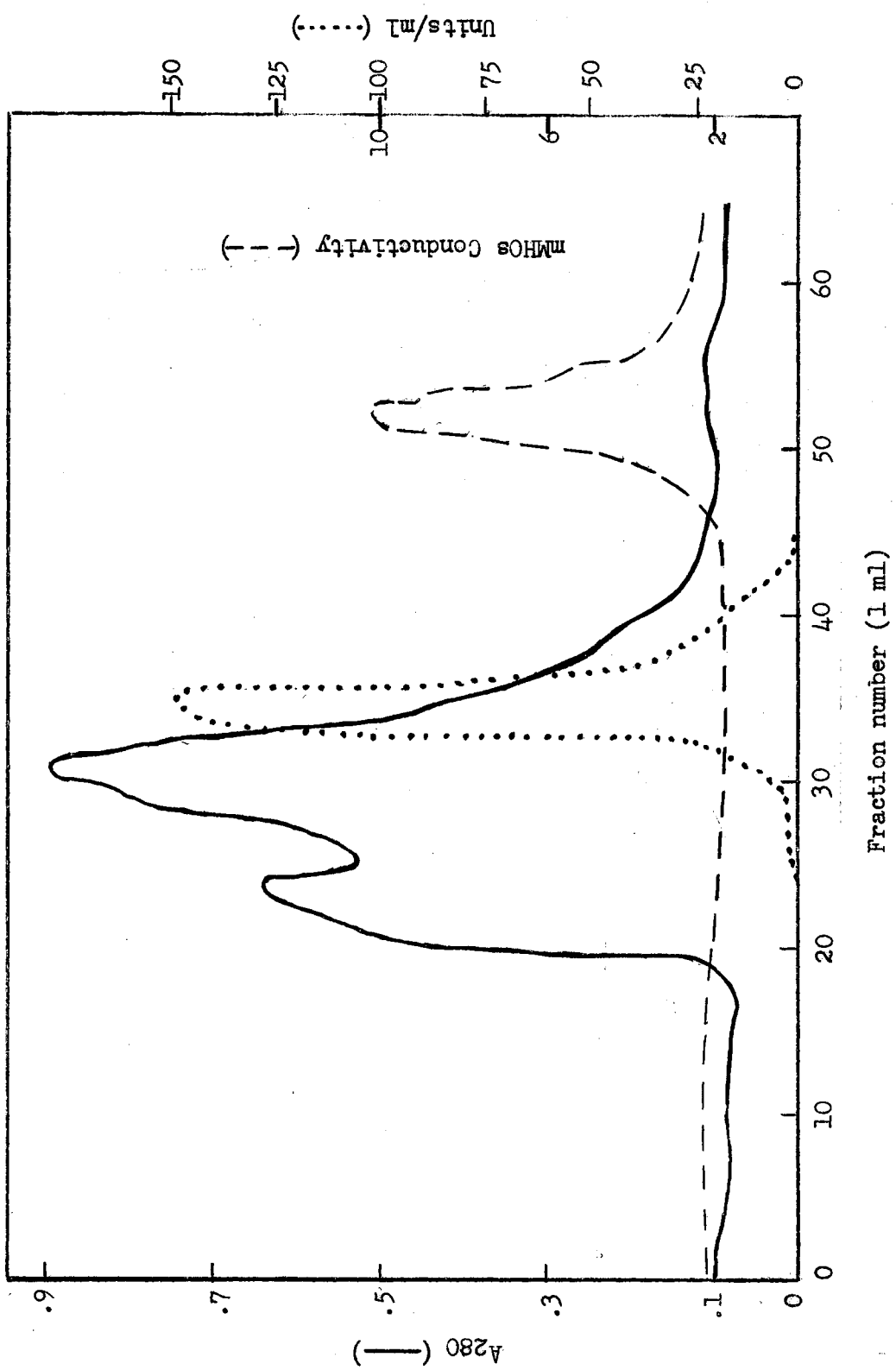

A_{280} (——)
Units/ml (·····)
M Potassium Phosphate (– – –)
Fraction number (7 ml)
0
.2
.4
.6
.8
.05
.10
.15
25
50
75
100
0
20
40
60
80
100
120
140
160

Figure 6

Elution Pattern of UDPGal 4-Epimerase from the Sephadex G-100 Column

One ml of concentrated enzyme solution (Step V) was passed through a 1 X 100 cm column of Sephadex G-100 equilibrated in 25 mM potassium phosphate, two mM mercaptoethanol, pH 7.6. Approximately one ml fractions were collected. Protein was monitored (A_{280}) on a Beckman DU spectrophotometer (——). Conductivity measurements (— —) were made on a conductivity meter and the peak between tubes 45 and 55 indicates where the ammonium sulfate came off. UDPGal 4-epimerase activity was determined by Assay I (·····).


A280 (——)
.9
.7
.5
.3
.1
0
10
20
30
40
50
60
Fraction number (1 ml)
mMHOs Conductivity (– – –)
Units/ml (·····)
150
125
100
75
50
25
0
10
6
2

CHAPTER VI

PROPERTIES OF UDPGAL 4-EPIMERASE

Stability Studies

The UDPGal 4-epimerase was found to be relatively unstable unless stored under the proper conditions, and then it could be stored for relatively long periods without substantial loss of activity. The enzyme was most stable when stored in a solution of high ionic strength. When the enzyme was stored as a concentrated solution after precipitation with ammonium sulfate, it was very stable. After desalting with BioGel P-10, activity was more rapidly lost upon storage, both at -20° and 0°.

In an experiment summarized in Table IV, the epimerase was stored in a variety of buffers after precipitation by ammonium sulfate in Step III. Activity was rapidly lost when the enzyme was kept at 0°, but was not when the enzyme was stored at -20°. Thawing and refreezing caused a loss of activity. The enzyme was more stable in phosphate buffer at pH 7.6 than at pH 7.0 and 8.2. It was also more stable in phosphate buffer than in Tris or glycine buffer or in deionized water.

The epimerase fractions collected from the DEAE cellulose and Sephadex G-100 columns rapidly lost activity and thus had to be precipitated immediately with ammonium sulfate. Attempts to purify the enzyme by use of heat (50° for two minutes) or low pH (5.6) fractionation failed because the enzyme loses activity rapidly at lower pH's and at higher temperatures.

TABLE IV

STABILITY OF UDPGAL 4-EPIMERASE UNDER VARIOUS STORAGE CONDITIONS

Storage Condition	Percent Activity Remaining		
	Stored four days at 1°	Stored two months at -20° thawed and refrozen four times	Stored four months at -20°
25 mM potassium phosphate, pH 7.6	52	88	96
50 mM potassium phosphate, pH 7.6	66	68	92
50 mM potassium phosphate, 2 mM mercaptoethanol, pH 7.0	55	48	90
50 mM potassium phosphate, 2 mM mercaptoethanol, pH 7.6	64	75	97
50 mM potassium phosphate, 2 mM mercaptoethanol, pH 8.2	29	58	91
0.2 M Tris, mM mercaptoethanol, 10 mM MgCl, mM EDTA, pH 7.4	9	13	90
0.2 M glycine, pH 8.7	45	0	90
deionized water	35	44	77

PCMB Effects

As discussed previously, PCMB has been shown to inhibit UDPGal 4-epimerase from yeast and calf liver, but does not inhibit the enzyme from E. coli. In the present experiments PCMB was shown to bind to the purified bovine mammary tissue epimerase but did not inhibit the enzymatic rate.

Assay I, using UDPGal 4-epimerase purified through Step V, was shown not to be inhibited by the addition of 10^{-9} moles of PCMB to the standard reaction mixture (Table V). The assay was inhibited by the addition of 10^{-8} moles PCMB due to the inhibition of UDPG dehydrogenase (50). A spectrophotometric assay for UDPG dehydrogenase was run by incubating the following reaction mixture in a 0.75 ml cuvette: 0.25 ml of 0.2 M glycine buffer, pH 8.7 (50 µmoles), 0.5 µmoles NAD^+, 0.02 µmoles UDPG, UDPG dehydrogenase (0.01 ml) and deionized water to give a final volume of 0.5 ml. The change in absorbance at 340 mµ was recorded on a Cary Model 14 spectrophotometer. This reaction was found to be inhibited by the addition of 0.5×10^{-9} mole of PCMB to the reaction mixture. When 0.01 ml of the UDPGal 4-epimerase used in Assay I was added to the reaction mixture before 10^{-9} moles of PCMB and then assayed, there was no inhibition of UDPG dehydrogenase (Table VI). This indicates that the UDPG dehydrogenase was protected from PCMB inhibition by its binding to the UDPGal 4-epimerase.

When the same experiment was conducted using more purified UDPGal 4-epimerase (through Step VI), Assay I was shown to be 50 percent inhibited by the addition of 3×10^{-10} moles of PCMB and completely inhibited by 10^{-9} moles (Table VII). The above UDPG dehydrogenase was

TABLE V

EFFECT OF PCMB ON ASSAY I USING STEP V EPIMERASE

Experiment No.	moles PCMB added	Units (μmole X 10^3 UDPG formed/min)	Percent inhibition
1	None	1.53	0
2	1×10^{-9}	1.45	5.2
3	5×10^{-9}	0.77	52.0
4	1×10^{-8}	0.16	89.5

TABLE VI

EFFECT OF PCMB ON UDPG DEHYDROGENASE AND PROTECTION BY UDPGAL 4-EPIMERASE (STEP V)

Experiment No.	Additions	μmole X 10^3 UDPG reacted	Percent inhibition
1	None	1.37	0
2	1×10^{-10} moles PCMB	1.13	19.7
3	5×10^{-10} moles PCMB	0.0	100
4	1×10^{-9} moles PCMB	0.0	100
5	1×10^{-9} moles PCMB 0.01 ml UDPGal 4-epimerase	1.28	6.6

75 percent inhibited by the addition of 3 X 10^{-10} moles of PCMB to the reaction mixture (Table VIII). When the same amount of UDPGal 4-epimerase used in Assay I was added to the UDPG dehydrogenase reaction mixture, the PCMB inhibition was reduced to 50 percent.

The same effect of protection of UDPG dehydrogenase by epimerase was observed in both experiments, but the effect was less pronounced with the more purified enzyme. This is probably due to the lower protein concentration in the more purified enzyme preparation (Step VI); thus less PCMB is bound.

The previous experiment did not show clearly whether PCMB did or did not have an inhibitory effect on UDPGal 4-epimerase since UDPG dehydrogenase is very sensitive to PCMB. To show whether PCMB inhibits UDPGal 4-epimerase activity, the following experiment was performed. A 0.5 ml sample of UDPGal 4-epimerase purified through Step V was incubated with five μmoles of PCMB (in 0.5 ml of 0.2 M glycine buffer, pH 8.7) for 30 minutes in an ice-bath. After incubation, the sample was passed slowly through a 1 X 15 cm column of BioGel P-10 equilibrated with 0.1 M phosphate, pH 7.6, to remove the unbound PCMB from the enzyme.

The PCMB is clearly shown to be separated from the enzyme in Figure 7 as indicated by the second A_{280} peak. The second 280 absorbing peak inhibited UDPG dehydrogenase (0.05 ml of tube 13 inhibited the standard assay by 50 percent). A control was run by passing an equal amount of enzyme through the same column without incubating with PCMB. From 80 units of enzyme put on the column in each case, 60 units were recovered from the control column run and 55 units were recovered after incubation with PCMB. These results indicate that PCMB-binding does not affect the activity of mammary tissue UDPGal 4-epimerase.

TABLE VII

EFFECT OF PCMB ON ASSAY I USING STEP VI EPIMERASE

Experiment No.	moles PCMB added	μmole X 10^3 UDPG formed/min	Percent inhibition
1	None	1.93	0
2	1×10^{-10}	1.33	31
3	3×10^{-10}	0.93	57
4	6×10^{-10}	0.52	73
5	1×10^{-9}	0.0	100

TABLE VIII

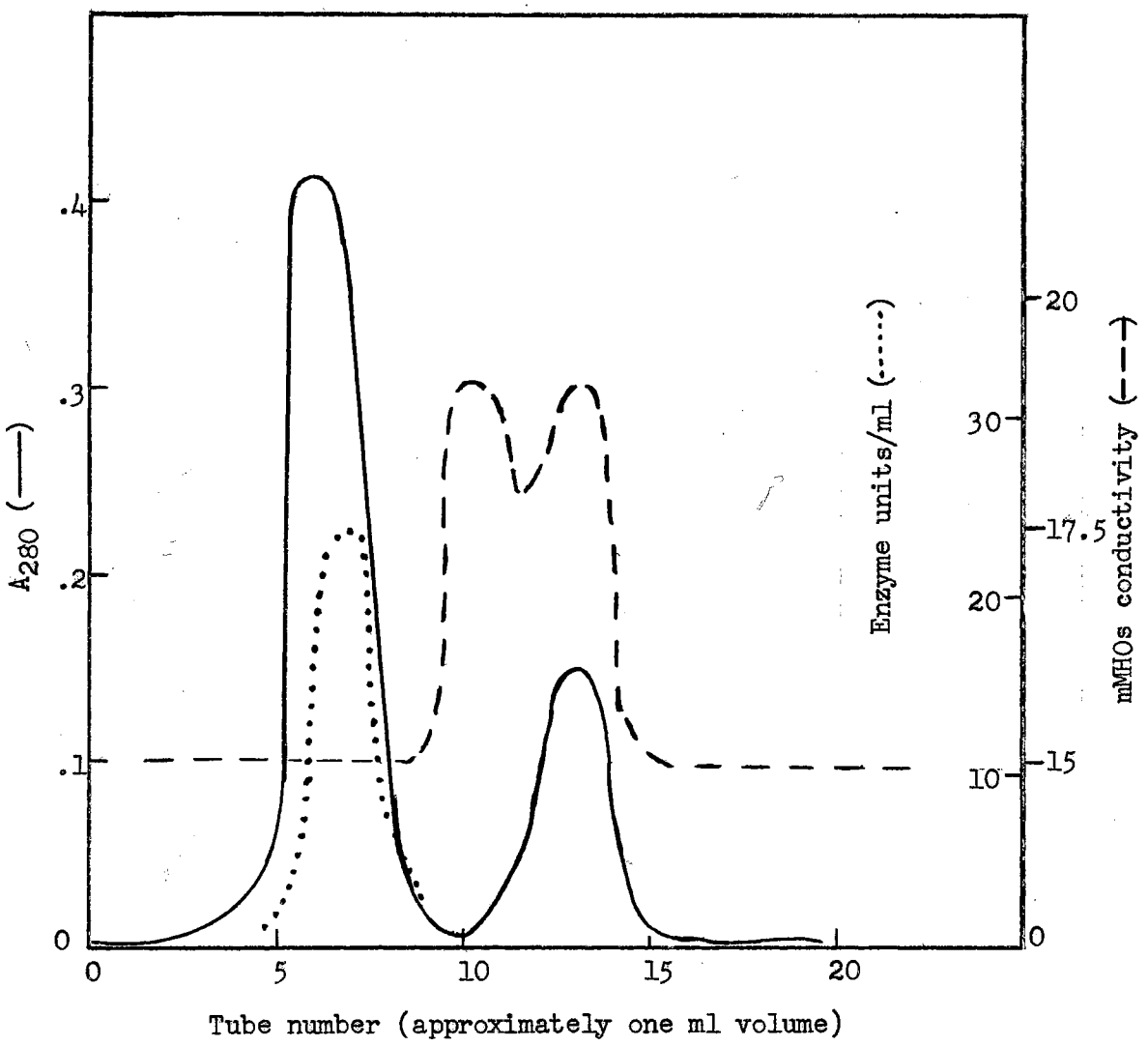
EFFECT OF PCMB ON UDPG DEHYDROGENASE AND PROTECTION BY UDPGAL 4-EPIMERASE (STEP VI)

Experiment No.	moles PCMB added	μmoles X 10^3 UDPG reacted/min	Percent inhibition	.01 ml UDPGal 4-epimerase added	
				μmoles X 10^3 UDPG reacted/min	Percent inhibition
1	None	4.44	0	--	--
2	1×10^{-10}	2.42	45.6	3.1	30.2
3	3×10^{-10}	1.17	73.7	2.2	50.5
4	1×10^{-9}	0.0	100	--	--

Figure 7

Separation of UDPGal 4-Epimerase and PCMB on BioGel P-10

One ml UDPGal 4-epimerase was passed through a 1 X 15 cm column of BioGel P-10 after incubation with five μmoles PCMB. The BioGel was equilibrated in 100 mM potassium phosphate buffer, pH 7.6, and approximately one ml fractions were collected. Each tube was measured for A_{280} (——), enzyme activity (·····) and conductivity (– – –). The second A_{280} peak was identified as PCMB since an aliquot from tube 13 would inhibit Assay I.


.4
.3
.2
.1
0
A_{280} (——)
0
5
10
15
20
Tube number (approximately one ml volume)
Enzyme units/ml (·····)
30
20
10
0
20
17.5
15
0
mMHOs conductivity (– – –)

UDPGal 4-Epimerase and NAD^+

Previous studies have shown that NAD^+ is required as a cofactor for UDPGal 4-epimerase, and is tightly bound to the enzyme isolated from microbial sources, but is loosely bound to the enzyme isolated from calf liver (23, 24, 25). To determine whether or not NAD^+ is tightly bound to bovine mammary tissue UDPGal 4-epimerase, the purified enzyme was assayed by Assay II with and without added NAD^+. As seen from Figure 8, NAD^+ added to the reaction mixture did not increase the reaction rate. Thus if NAD^+ is required for catalysis, it must be tightly bound to the enzyme.

The bound NAD^+ has been removed with charcoal from epimerases isolated from other sources, but no reactivation of the enzyme by the addition of NAD^+ has been shown. An attempt was made to remove NAD^+ with charcoal from the enzyme and reactivate the enzyme by the addition of NAD^+.

The epimerase, purified through step 5, was diluted three to one with 25 mM potassium phosphate buffer, five mM mercaptoethanol, pH 8.0. Two ml of the enzymes were stirred with ten mg of activated charcoal for about ten minutes at 0°. The charcoal was centrifuged and the supernatant was assayed by Assay II, with and without the addition of 10^{-7} moles of NAD^+. Successive additions of 10, 10, and 25 mg of charcoal were made and the supernatant solution was assayed after each addition.

As can be seen from Table IX, the epimerase was reactivated by about 35 percent upon the addition of NAD^+. After the addition of 55 mg of charcoal, no activity was obtained without the addition of NAD^+ to

Figure 8

Effect of Added NAD^+ on Assay II for UDPGal 4-Epimerase

UDPGal 4-epimerase purified through Step VI was assayed by Assay II with (o) and without (•) the addition of 0.10 μmole NAD^+ to the standard incubation mixture. The data presented are the combination of two separate experiments.

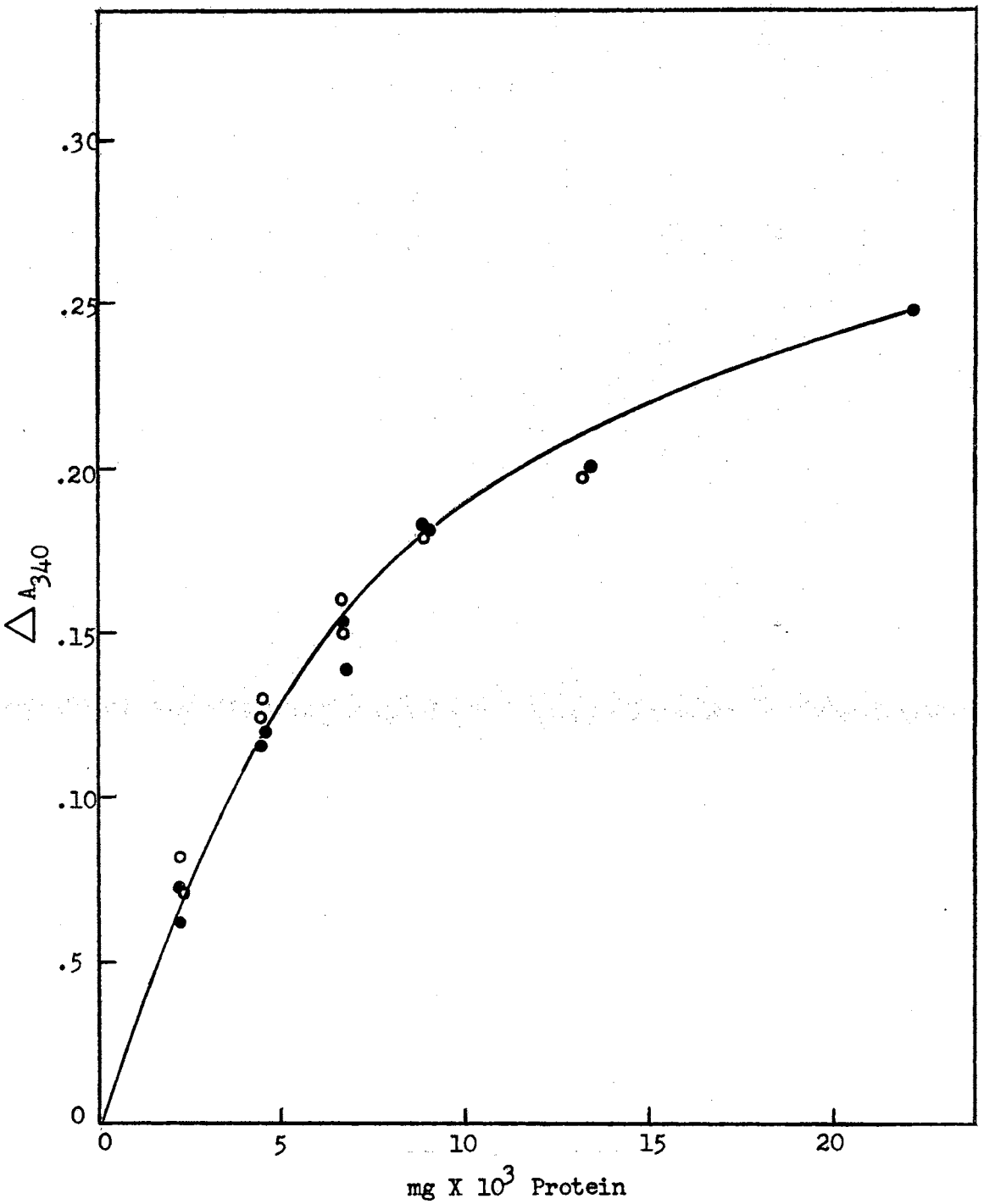

.30
.25
.20
.15
.10
.5
0
ΔA_{340}
0
5
10
15
20
mg X 10^3 Protein

TABLE IX

INACTIVATION OF UDPGAL 4-EPIMERASE BY CHARCOAL AND REACTIVATION BY THE ADDITION OF NAD^+

Total mg of charcoal added	μmole X 10^2 UDPG formed		Percent reactivation
	No NAD^+	10^{-7} moles NAD^+ added	
0	---	2.96	---
10	2.00	2.66	33
20	1.74	2.39	36
30	1.25	1.37	9.5
55	0	.47	---

the reaction mixture. The data support the view that NAD^+ was tightly bound to the enzyme but apparently could be removed by activated charcoal. The charcoal-treated enzyme could be reactivated by the addition of NAD^+.

pH Optimum

The effect of pH on UDPGal 4-epimerase activity was determined by using Assay I in a buffer which was 0.1 M with respect to both glycine and Tris. The buffer solution was adjusted to various pH values (7.0 to 9.5) by the addition of HCl or KOH. The reaction rate in this buffer at pH 8.7 was the same as the rate obtained in the standard 0.2 M glycine buffer, pH 8.7. UDPG dehydrogenase was used at concentrations so that it was not rate limiting (determined by assay); thus larger amounts were used at the higher and lower pH values where the enzyme is less active (50). The UDPGal 4-epimerase used in this study was purified through Step 5. The pH optimum range was found to be between 8.5 and 9.0 as shown in Figure 9.

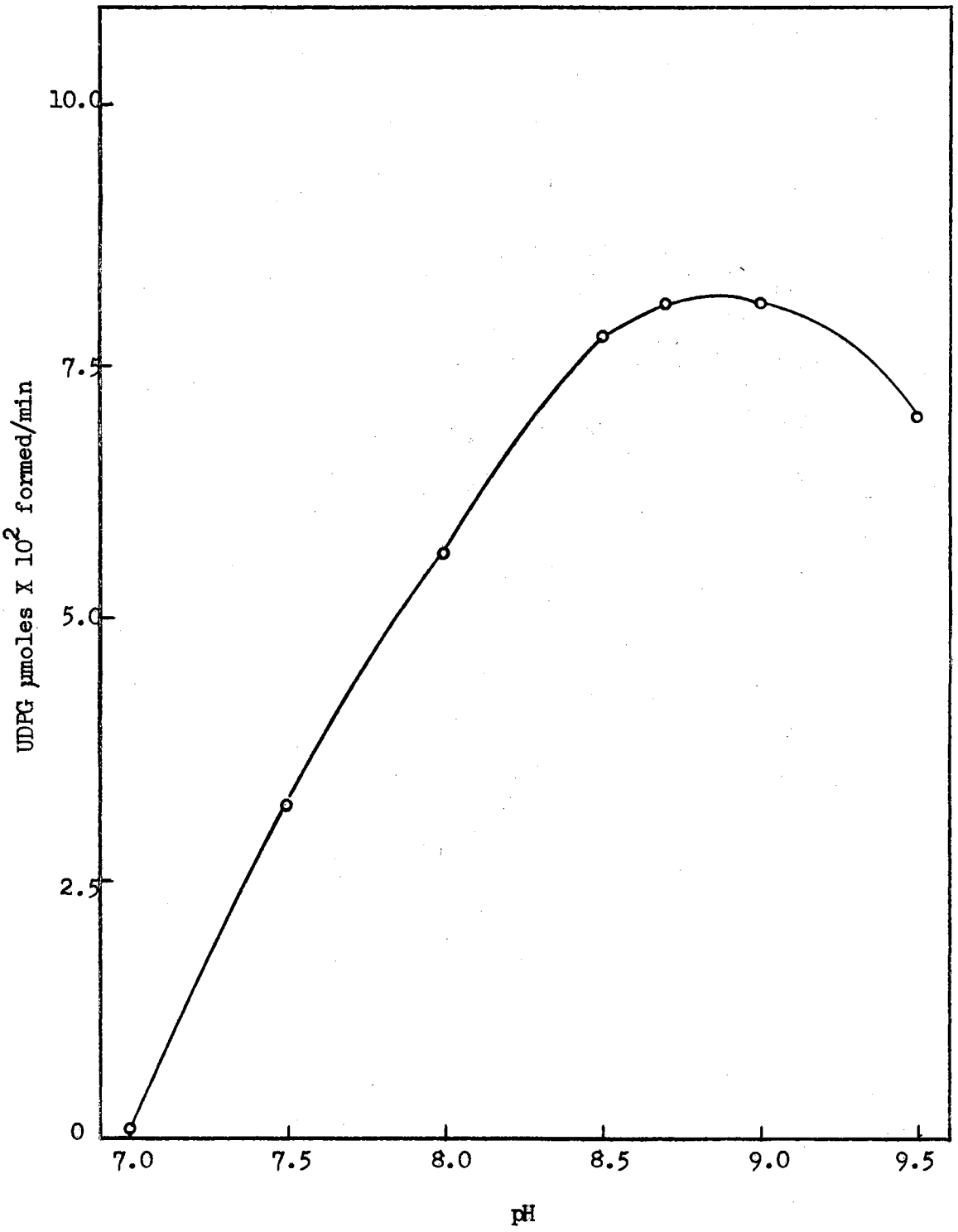
Kinetic Parameters

The Km for UDPGal and UDPG was determined from Lineweaver-Burk plots (64) of the reciprocal of the rate against the reciprocal of the substrate concentration. UDPGal was repurified on a Dowex 1 column as previously described and the concentration was determined from the extinction coefficient ($E_{\mu M} = 6.2 \times 10^{-3}$) at 260 mμ. Assay I was used to determine the Km of UDPGal. Rates were determined with an optimal amount of enzyme and varying amounts of substrate. A plot of initial rate <u>versus</u> UDPGal concentration is shown in Figure 10 and the Lineweaver-

Figure 9

pH Optimum Curve

UDPGal 4-epimerase activity was measured by Assay I at different pH values in a buffer which was 0.1 M with respect to both glycine and Tris. UDPG dehydrogenase was used at concentrations which were not rate-limiting at the pH indicated.


10.0
7.5
5.0
2.5
0
UDPG μmoles X 10^2 formed/min
7.0
7.5
8.0
8.5
9.0
9.5
pH

Burk plot is shown in Figure 11. The Km for UDPGal was calculated to be 5.7×10^{-5} M.

Assay II was used to determine the Km of UDPG. The rate of UDPGal formation was calculated by determining the amount of UDPG remaining in the reaction mixture after incubation with UDPGal 4-epimerase for five minutes and subtracting this value from the initial amount of UDPG. A plot of initial rate of UDPGal formation versus substrate concentration is shown in Figure 12 and the reciprocal plot is shown in Figure 13. The Km for UDPG was found to be 2.3×10^{-3} M.

Figure 10

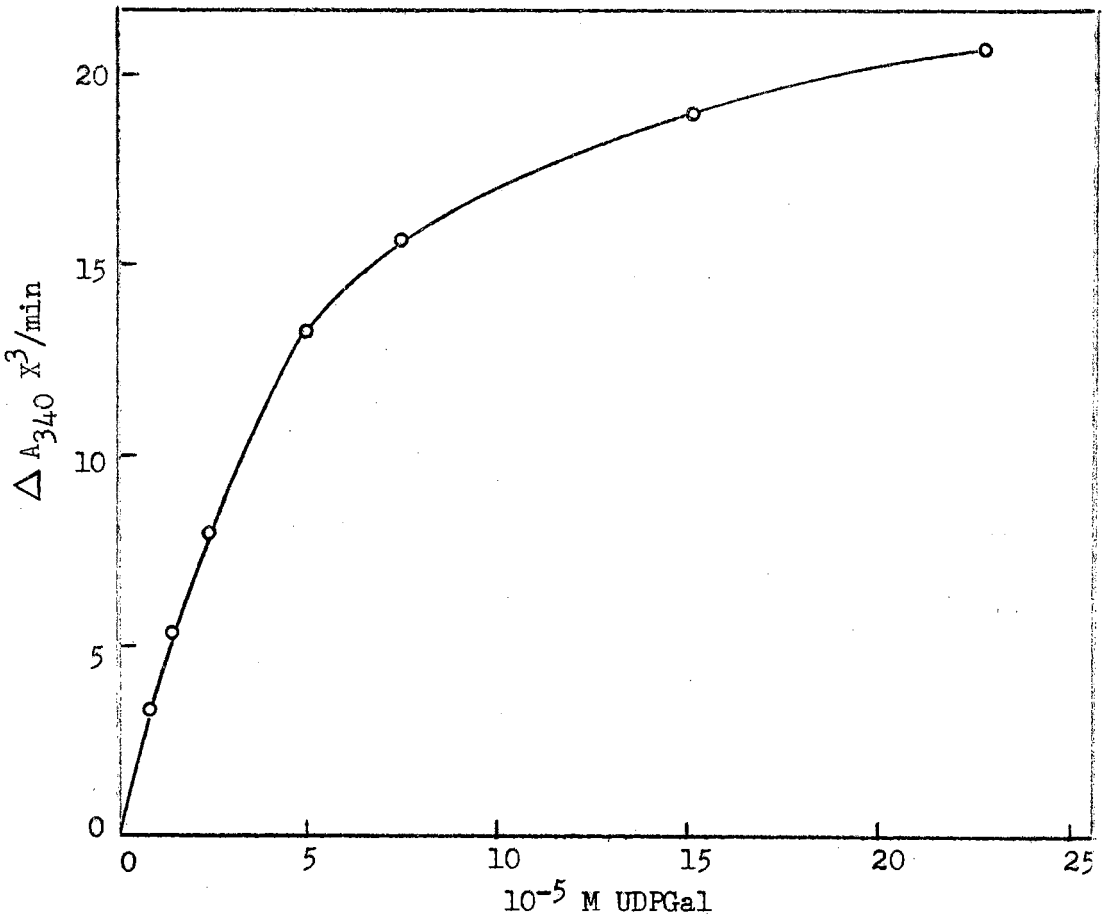
Effect of UDPGal Concentration on Initial Rate of UDPGal 4-Epimerase

UDPGal 4-epimerase purified through Step VI was assayed by Assay I using varying concentrations of UDPGal. Initial rate is plotted against UDPGal concentration.

Figure 11

Lineweaver-Burk Plot for Determining the Km of UDPGal

The reciprocal velocity is plotted against the reciprocal UDPGal concentration using the data obtained in Figure 10. The Km was determined by extrapolating to the base line.


20
15
10
5
0
ΔA_{340} X^3/min
0
5
10
15
20
25
10^{-5} M UDPGal

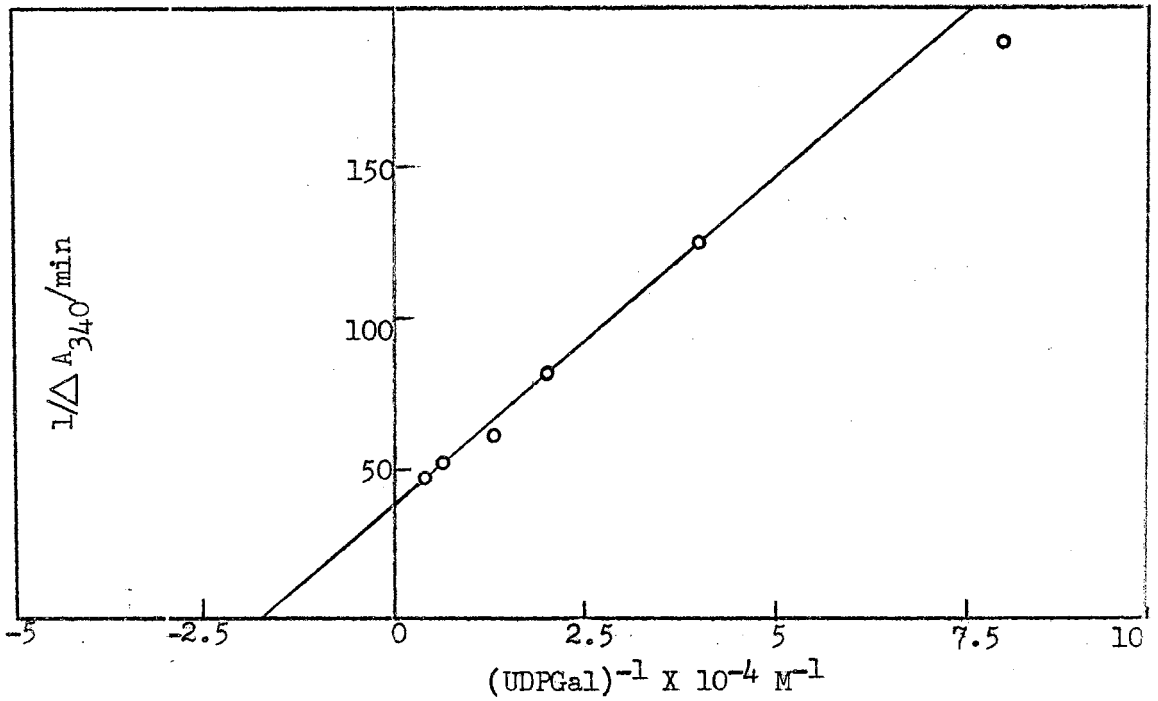

150
100
50
$1/\Delta A_{340}$/min
-5
-2.5
0
2.5
5
7.5
10
$(UDPGal)^{-1}$ X 10^{-4} M^{-1}

Figure 12

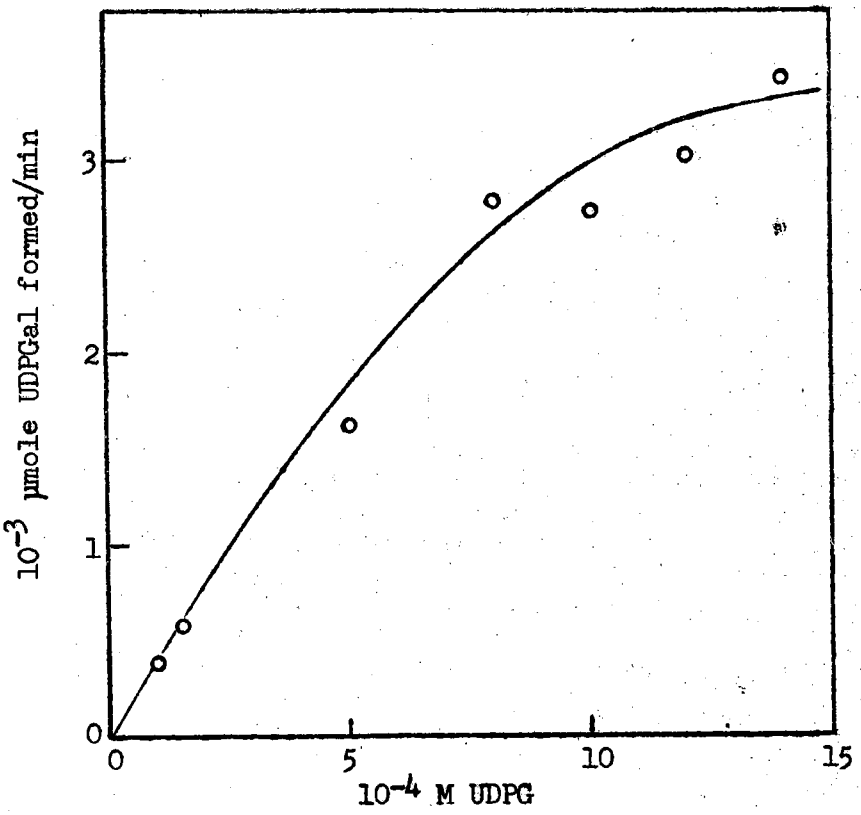
Effect of UDPGal Concentration on Initial Rate of UDPG 4-Epimerase

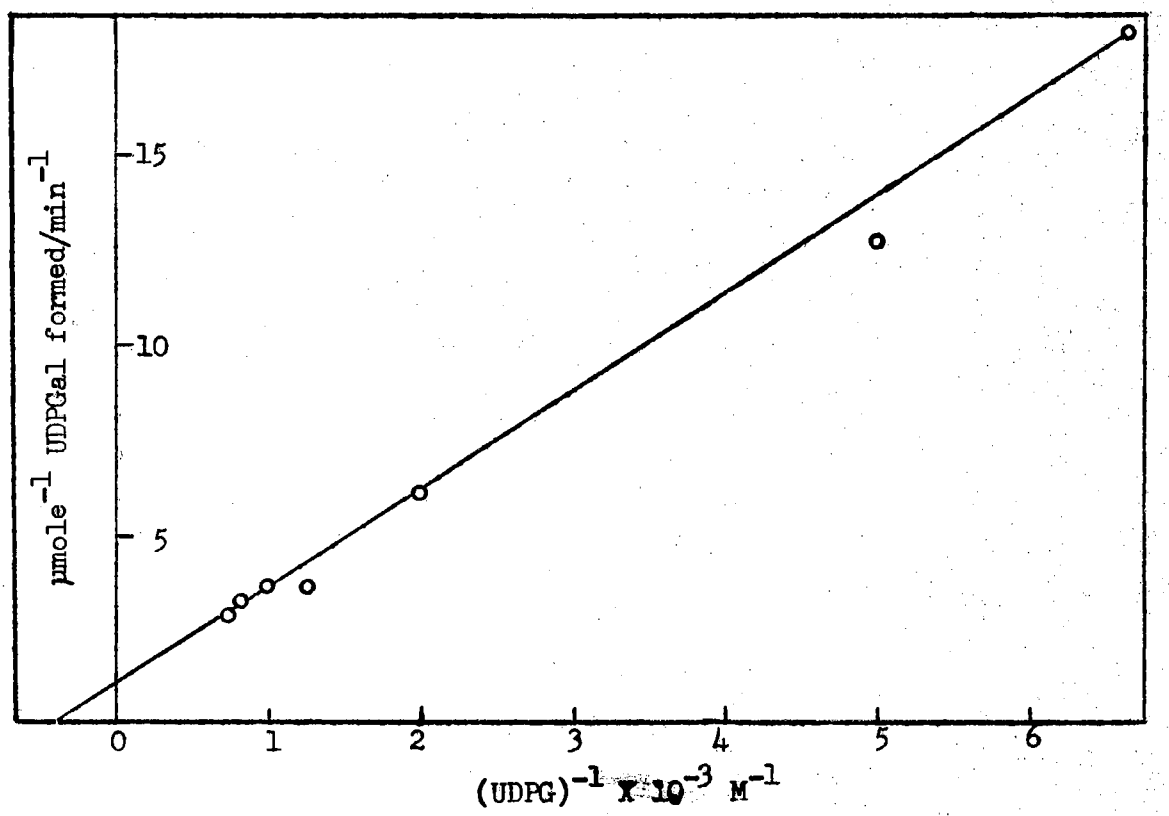
Varying amounts of UDPG were incubated with epimerase from Step VI. The amount of UDPG remaining after incubation was calculated from the total A_{340} change in Assay II. The amount of UDPGal formed was calculated by subtracting the amount of UDPG remaining from the amount in the initial reaction mixture.

Figure 13

Lineweaver-Burk Plot for Determining the Km of UDPG

The reciprocal velocity versus the reciprocal UDPG concentration was plotted using the values from Figure 12. The Km for UDPG was determined by extrapolating to the base line.


10^{-3} µmole UDPGal formed/min
0
1
2
3
0
5
10
15
10^{-4} M UDPG


µmole^{-1} UDPGal formed/min^{-1}
5
10
15
0
1
2
3
4
5
6
(UDPG)$^{-1}$ X 10^{-3} M^{-1}

CHAPTER VII

DISCUSSION

Methods used in attempts to synthesize UDPGal were not entirely as satisfactory as reported in the literature, but sufficient quantities of UDPGal were synthesized for use in the enzymatic assays. The enzymatic method of Anderson et al. (52) yielded no purified UDPGal because it could not be separated on the DEAE cellulose column from other nucleotides present in the reaction mixture. Difficulties were also encountered in keeping all of the enzymes of the yeast enzyme system active. The chemical synthesis of UDPGal by the Michelson procedure (53) was unsuccessful because it was difficult to dissolve the UMP salt. This may have been due to the fact that the reactants and the solvents were not completely anhydrous, even though the solvents were dried with molecular sieves. However, the molecular sieves were used directly as purchased and recent experience has shown that they must be carefully dried prior to use. The method of Moffatt and Khorana (54, 55) resulted in sufficient quantities of UDPGal, but yields were reduced because of the difficulty of dissolving the Gal-1-P amine salt in pyridine. Moffatt (60) has recently pointed out that it is most essential to have the amine salts in solution prior to condensation.

The isolation procedure for UDPGal 4-epimerase from acetone powder resulted in a 20 to 30-fold purification with 13 percent of the total enzyme units recovered. The individual steps in the procedure resulted

in no striking purification. The protamine sulfate fractionation did not result in a large purification, but did remove nucleic acids and hence the protein solution was easier to work with in subsequent steps. The Sephadex G-100 column gave a very sharp UDPGal 4-epimerase peak, but resulted in the loss of 60 percent of the total units, possibly due to the low ionic strength and the time required to run the column (two to three days). For future purifications, it would be recommended that the Sephadex G-100 column be equilibrated with a buffer of higher ionic strength, e.g. 100 mM potassium phosphate, pH 7.6. The enzyme as purified is not a homogeneous protein, as can be seen from the profile obtained from the Sephadex G-100 column (Figure 6).

The properties of the UDPGal 4-epimerase isolated from mammary tissue were similar in many respects to the properties of the epimerase isolated from other sources which are summarized in Table II. The mammary tissue epimerase was found to be stable when stored at -20° in a solution of high ionic strength, but rather unstable under the usual isolation conditions. The optimum pH range was from 8.5 to 9.0, which is similar to the range found for the enzyme isolated from other sources. Data were obtained which indicate that the mammary tissue epimerase would bind PCMB but that PCMB did not inhibit the enzyme. Some caution must be used in interpreting the binding data since the more purified enzyme preparation apparently bound less PCMB than the less purified enzyme preparation. The epimerase isolated from E. coli was not inhibited by PCMB, but the enzyme from yeast and calf liver were inhibited. The present data are consistent with the view that NAD^+ is tightly bound to the mammary tissue UDPGal 4-epimerase. It has previously been found that NAD^+ is tightly bound to the epimerase

isolated from yeast and bacteria, but loosely bound to the enzyme isolated from calf liver. A Km value of 5.7×10^{-5} M for UDPGal was found for the mammary tissue enzyme, which is almost the same as the Km found for the calf liver epimerase, but slightly lower than the values obtained for the enzyme isolated from other sources. The Km for UDPG was found to be 2.3×10^{-3} M. This value is close to that found for the E. coli epimerase, but is much higher than the value found for the epimerase isolated from calf liver.

CHAPTER VIII

SUMMARY

UDPGal was synthesized by the method of Moffatt and Khorana (54, 55). UDPGal 4-epimerase was purified approximately 30 fold from bovine mammary acetone powder. The enzyme was found to be stable when stored at -20° in a solution of high ionic strength. PCMB is apparently bound by the enzyme but does not inhibit activity. Data indicate that NAD^+ is bound to the purified enzyme. The enzyme can be inactivated by charcoal and can be reactivated by NAD^+. The enzyme has an optimum pH range of 8.5 to 9.0. The Km for UDPGal and UDPG were found to be 5.7×10^{-5} M and 2.3×10^{-3} M, respectively.

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