I. CHEMICAL SYNTHESIS OF LACTOSE-1-PHOSPHATE AND GALACTOSE 1,6-DIPHOSPHATE

II. INDUCTION OF TYROSINE TRANSAMINASE IN ISOLATED RAT LIVER CELLS

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PART I

CHEMICAL SYNTHESIS OF LACTOSE-1-PHOSPHATE AND GALACTOSE 1,6-DIPHOSPHATE

CHAPTER I

INTRODUCTION

The biological importance of lactose-1-phosphate and galactose 1,6-diphosphate is questionable. Lactose-1-phosphate was proposed as an intermediate in the biosynthesis of lactose (1,2,3), but other investigators have not verified these findings. In order to investigate a possible biological role for lactose-1-phosphate it is necessary to have adequate amounts of the compound available and to date it has been difficult to synthesize this compound.

There appears to be no evidence for the existence of galactose 1,6-diphosphate in biological materials though glucose 1,6-diphosphate and fructose 1,6-diphosphate are found in red blood cells (4). However, it would appear that galactose 1,6-diphosphate might be an inhibitor of the phosphoglucomutase reaction since glucose 1,6-diphosphate is required for reaction. The purpose of synthesizing galactose 1,6-diphosphate was to test it as an inhibitor of phosphoglucomutase.

Previous methods (5,6) for the chemical synthesis of lactose-1-phosphate were difficult. There is no report on the chemical synthesis of galactose 1,6-diphosphate. Recently, a new and simple method became available for the synthesis of various glycosyl-1-phosphates and this method was used for the synthesis of lactose-1-phosphate and galactose 1,6-diphosphate.

CHAPTER II

LITERATURE REVIEW

Methods for the Synthesis of Lactose-1-Phosphate

In studies on the biosynthesis of lactose by the mammary gland, Malpress et al. (1) postulated lactose-1-phosphate as an intermediate in the synthesis. Lactose-1-phosphate also was suggested by Rogosa (2) in 1948 as an intermediate in lactose fermentation by yeast. He reasoned that if preliminary hydrolysis of lactose in a fermentation did not occur, then it was necessary to postulate reactions analogous to that of Price, Cori, and Colowick (7).

Gander et al. (3) observed phosphoglucomutase (EC 2.7.5.1.), and galactosyl transferase (EC 2.4.1.) activities in bovine mammary gland of lactating cows. Observation of the galactosyl transferase activity in mammary gland which formed lactose-1-phosphate from UDPgalactose and glucose-1-phosphate led them to propose a pathway of lactose synthesis from glucose:

$$2\ \text{Glucose} \xrightarrow[\text{Hexokinase}]{\text{ATP} \rightarrow \text{ADP}} 2\ \text{Glu-6-P} \xrightarrow{\text{Phosphoglucomutase}} 2\ \text{Glu-1-P}$$

$$\text{UTP} + \text{Glu-1-P} \xrightarrow{\text{UDPGlu Pyrophosphorylase}} \text{UDPGlu} + \text{PP}$$

$$\text{UDPGlu} \xrightarrow{\text{UDPGal-4-Epimerase}} \text{UDPGal}$$

$$\text{UDPGal} + \text{Glu-1-P} \xrightarrow{\text{Galactosyl Transferase}} \text{UDP} + \text{Lactose-1-Phosphate}$$

Lactose-1-Phosphate $\xrightarrow{\text{Phosphatase}}$ Lactose .

However they did not present any evidence for the hydrolysis of lactose-1-phosphate to lactose.

In 1952, Reithel and Young (5) prepared α-lactose-1-phosphate by the method of Cori (8), employing trisilver phosphate as the phosphorylating agent, but yields were low. They also attempted to prepare the compound by the procedure described by Posternak (9) in which silver diphenyl phosphate was used as a phosphorylating agent. Again the yields were low and the product was impure. However the preparation of the β-form of the phosphate was successfully obtained by the use of monosilver phosphate as a phosphorylating agent (10).

Sasaki and Taniguchi (6) prepared octa-O-acetyl-β-lactose, m.p. 140-141° and treatment with acetic acid containing HBr to 80 % saturation yielded hepta-O-acetyl-α-lactosyl bromide. Addition of silver diphenyl phosphate in dry benzene and subsequent hydrogenation gave the product as hepta-O-acetyllactosyl phosphate. Addition of 10 % $Ba(OAc)_2$ formed a precipitate, which was then removed. The filtrate was treated with 5 N NaOEt to precipitate α-lactose-1-phosphate. Treatment of hepta-O-acetyl-α-lactosyl bromide with silver dibenzyl phosphate gave β-lactosyl phosphate·5 H_2O. However, they did not succeed in crystallizing the potassium salt.

Chemical Synthesis of Aldose-1-phosphates

There are several methods which have been devised for the chemical synthesis of sugar phosphates. In 1937, Cori, Colowick, and Cori (8) reported the synthesis of glucose-1-phosphate by the reaction of

acetobromoglucose and silver phosphate to produce the tertiary ester, tri-(α-D-glucose-1-)-phosphate. To obtain glucose-1-phosphate, the compound required deacetylation and removal of two sugar residues from the molecule. The deacetylation was carried out with 5 N HCl to give the product, glucose-1-phosphate, which appeared to be identical with the natural product with regard to chemical and physiological properties. The reaction of acetobromoglucose and silver phosphate permits phosphorylation only in the C-1 position of the glucose molecule. Also the acetobromoglucose has a pyranoid structure and thus the synthetic product was assumed to be α-D-glucose-1-phosphate (8). The following year, Colowick (11) prepared α-D-galactose-1-phosphate and α-D-mannose-1-phosphate by the same method used for the synthesis of glucose-1-phosphate. The specific rotation for both sugar phosphates showed that they were the α-anomers. Usually this method is used when the α-acetobromoaldose is available in great quantities. Crystalline acetobromogalactose was prepared as described by Ohle et al. (12).

Posternak (9) presented a new method for the synthesis of aldose-1-phosphates. In the synthesis of α-D-glucose 1,6-diphosphate, the success of the method depends on treating an α-acetobromoaldose with silver diphenylphosphate as the phosphorylating agent. The phenyl groups are removed from the intermediate compound by catalytic hydrogenation and the acetyl groups are removed by base hydrolysis. Thus an aldose-1-phosphate is formed, which in the case of glucose 1,6-diphosphate, is primarily the α-anomer.

Posternak (13) used the silver diphenyl phosphate method to synthesize α-D-glucose-1-phosphate and α-D-galactose-1-phosphate. Silver diphenylphosphate condenses with α-D-acetobromo sugars without inversion

which is markedly different from other monosilver phosphate derivatives (16) and dibenzylphosphate (14). With this method, Posternak was able to synthesize α-D-glucose-1-phosphate and α-D-galactose-1-phosphate as the crystalline potassium salts, in 37 % and 44 % overall yields, respectively. These yields are four to six times higher than the best reported yields obtained by the trisilver phosphate method. Another procedure which was applied by the Posternak (13) was the treatment of D-glucose-2,3,4,6-tetraacetate with diphenyl chlorophosphate in pyridine. However this method gave inversion and the final yield (10 %) was much lower than that obtained by the silver diphenyl phosphate procedure.

The "monosilver phosphate" method of preparing β-D-aldose-1-phosphates from α-1-bromo-tetraacetylglucose was devised by Reithel (10) in 1945. This method is generally applicable to the synthesis of the β-isomer of hexose-1-phosphates.

Putman and Hassid (15) used Reithel's method (10) to prepare β-D-glucose-1-phosphate, β-D-galactose-1-phosphate as the cyclohexylammonium salts contaminated with less than 0.1 % of the α-anomers as shown by the enzymatic assay (16). Putman and Hassid (15) concluded that the ring configuration is maintained in the phosphorylated sugar but an inversion occurred at the C-1 position resulting in the 1,2-trans-glycosyl ester as the principal product when the bromine is replaced by phosphate using the monosilver phosphate method described by Reithel (10).

In recent years, cyclohexylamine has been employed extensively for the preparation of crystalline salts of phosphorylated sugars. As a result, deoxyribose-1-phosphate (17) and D-fructose 1,6-diphosphate (18) were obtained readily as the crystalline cyclohexylammonium salts. A number of other phosphorylated sugars also have been crystallized as

pure cyclohexylammonium salts by various investigators (19,20,21,22).

In 1962, a very useful and widely adopted method for glycosyl phosphate synthesis was developed by MacDonald (23). MacDonald (23) prepared glycosyl phosphates by an entirely different procedure which involves the phosphorylation of the fully acetylated sugar by anhydrous phosphoric acid *in vacuo*. The acetyl group on carbon one is replaced by a phosphate group with the evolution of acetic acid. Clearly, with β-D-glucose pentaacetate and β-D-galactose pentaacetate, MacDonald (23) obtained the α-1-phosphate as crystalline potassium or cyclohexylammonium salts in yields of 30 % and 35 % respectively. Further removal of the acetyl groups was obtained with lithium hydroxide which also precipitated excess phosphoric acid as the insoluble lithium salt.

The MacDonald method was applied by O'Brien (24), with slight modification, in the synthesis of N-acetyl-α-D-glucosamine-1-phosphate and N-acetyl-α-D-galactosamine-1-phosphate. O'Brien (24) reported that the highest yields were obtained by increasing the molar ratio of phosphoric acid to pentaacetyl-α-D-glucosamine from 4:1 to 8:1 and heating the reaction mixture at a higher temperature (83^{o} instead of 50^{o}) for a shorter time (45 min. instead of 2 hours). Under different test conditions the overall yield varied from 7 % to 33 %. Separation of the anomers was attained by ion-exchange chromatography.

In 1966, MacDonald (25) reported several modifications of the original procedure, which was already widely adopted in conjunction with the preparation of sugar nucleotides employing the general method (26) based on the reaction of a glycosyl phosphate with a nucleotide 5'-phosphoromorpholidate (33). MacDonald (25) investigated modifications of the fusion technique using a solvent and using polyphosphoric acid

and also some aspects of the effect of anomeric configuration on the reaction. MacDonald (25) reported that they improved the yields with compounds which have 1,2 *trans* - diequatorial configuration. By using a molar ratio of phosphoric acid to sugar acetate of 8:1, with stirring for 2 hours at 50° *in vacuo*, they obtained α-D-glucose-1-phosphate and α-D-galactose-1-phosphate in 60 % yield from the corresponding β-pentaacetates of glucose and galactose. Sugars which have a 1,2 *cis*-configuration did not react well at 50° and final yields of the products were poor. However, it was shown that the reaction of β-glucose pentaacetate with phosphoric acid at 85° for 45 min. gave α-D-galactose-1-phosphate in a purified yield of 27 % (28). MacDonald (25) also tried "105 %" phosphoric acid reasoning that the water content in phosphoric acid may influence the course of reaction of an acetylated sugar. However only a 46 % yield of glucose-1-phosphate was obtained with this reagent. This is approximately the same yield obtained with anhydrous phosphoric acid.

Chemical Synthesis of Aldose 1,6-Diphosphates

Leloir *et al.* (29,30) reported that phosphoglucomutase (EC 2.7.5.1.) requires a coenzyme which was postulated to be glucose 1,6-diphosphate because of the acid lability of a part of its phosphorous and the formation of glucose-6-phosphate by hydrolysis. In 1949, Posternak (9) attempted to chemically synthesize glucose 1,6-diphosphate by the introduction of a phosphate group into suitable derivatives of glucose-6-phosphate or glucose-1-phosphate.

Posternak (9) concluded that the former procedure was better then the latter. Condensation of α-2,3,4-triacetyl-6-

diphenylphosphonoglucosyl bromide (I) with silver diphenyl phosphate, followed by catalytic hydrogenation and alkaline hydrolysis, produced a better yield of α-glucose 1,6-diphosphate than the condensation of the bromo compound (I) with trisilver phosphate, followed by catalytic hydrogenation and partial acid hydrolysis. The success of the reaction depends on treating an α-acetobromo-aldose with silver diphenylphosphate. In the case of glucose 1,6-diphosphate, the product is mainly the α-form. Other aldose-1-phosphates can be formed by this method.

Posternak and Rosselet (31) reported in 1953 that α-mannose 1,6-diphosphate was synthesized from 1,2,3,4-tetraacetyl-6-diphenylphosphonyl-β-D-mannose, which was prepared by the condensation of 1,2,3,4-tetraacetyl-β-D-mannose with diphenyl phosphonyl chloride in pyridine. By the Pacsu method (32), 2,3,4-triacetyl-6-diphenylphosphonyl-α-mannosyl chloride (31) was obtained. Finally α-D-mannose, 1,6-diphosphate was obtained by refluxing 2,3,4-triacetyl-6-diphenylphosphonyl-α-mannosyl chloride in benzene with silver diphenyl phosphate, followed by hydrogenation of the product.

Khan et al. (33) adopted the MacDonald technique and were able to synthesize α-D-glucose 1,6-diphosphate from glucose-6-phosphate by a simple two-step chemical reaction which involved phosphorylation of β-1,2,3,4-tetraacetyl glucose-6-phosphate with anhydrous phosphoric acid. The yield of the cyclohexylammonium salt of α-D-glucose 1,6-diphosphate was between 20 and 25 %.

Recently, Buck (34) reported a similar synthesis. Glucose-6-phosphate was acetylated with acetic anhydride and sodium acetate to yield 1,2,3,4-tetraacetyl glucose-6-phosphate which was isolated in yields of 50-55 % as the crystalline methanolate. Phosphorylation of

1,2,3,4-tetraacetyl glucose-6-phosphate with MacDonald's method (23), followed by deacetylation, gave glucose 1,6-diphosphate in yields of 30-35 %. The product contained α- and β-anomers in a ratio of 9:1 as determined by optical rotary measurements.

In the present paper, it is proposed to synthesize α-D-galactose 1, 6-diphosphate by adopting the procedure used by Khan et al. (33) and to prepare galactose-6-phosphate by the method of Levene (35,36).

CHAPTER III

EXPERIMENTAL PROCEDURES

Materials

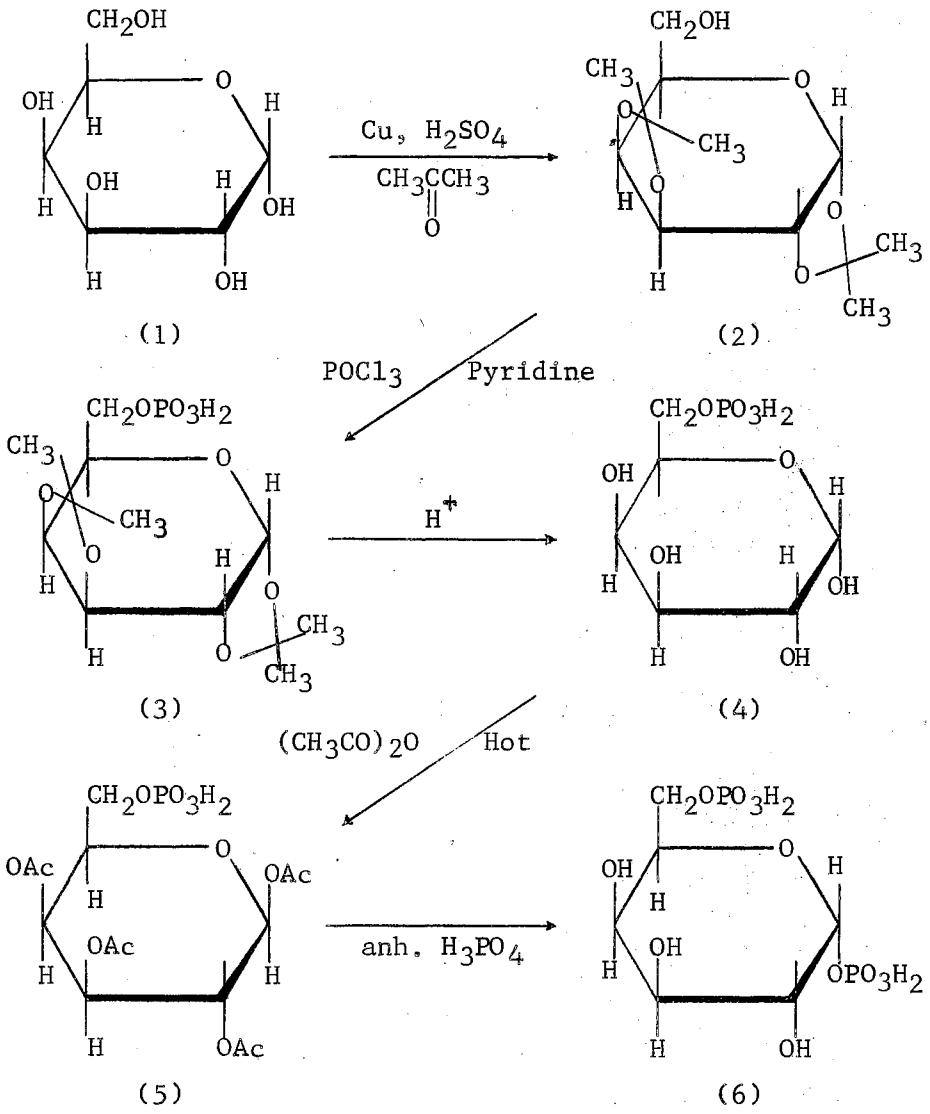
Lactose was obtained from Mann Research Laboratories, Inc.. Phosphoric acid was from Matheson Coleman and Bell. Dowex 50W x 8 cation exchange resin (50-100 mesh) was obtained from Bio Rad Laboratories. Phosphoglucomutase (from rabbit muscle), glucose-6-phosphate dehydrogenase, and glucose-1-phosphate (Grade V) were from Sigma Chemical Company. Glucose 1,6-diphosphate was prepared by Dr. Khan (33) in this laboratory previously. All other chemicals used were of reagent quality.

Methods

Acetylation of lactose was performed by the procedure of Hudson and Johnson (35). Galactose-6-phosphate was prepared according to Levene *et al.* (36,37), preceded by the preparation of diacetone galactose (35). MacDonald's method (23) was used to phosphorylate β-lactose octaacetate and β-1,2,3,4-tetraacetyl galactose-6-phosphate to make lactose-1-phosphate and galactose 1,6-diphosphate. Inorganic, acid labile and heat stable phosphate were determined by a modified Fiske-Subbarow's method (38). The amount of reducing sugar was measured by Nelson's method (39). Infrared spectra were obtained on Beckman IR5A Infrared Spectrophotometer and Perkin-Elmer 457 Grating Spectrophotometer. A

scheme for the synthesis of galactose 1,6-diphosphate is presented in Figure 1.

The inhibition of galactose 1,6-diphosphate on the phosphoglucomutase reaction was determined by the coupled assay method of Ray et al. (41). A mixture of 0.7 ml of pH 7.4 histidine-Tris buffer (0.08 M histidine-0.025 M Tris-chloride and 3mM magnesium sulfate), 0.1 ml of serum albumin (0.4 mg/ml), 0.001 - 0.01 ml of glucose 1,6-diphosphate solution (15 mM), and 0.005 ml of phosphoglucomutase (1 to 100 dilution) was placed in a cuvette with a 1-cm light path. The mixture was equilibrated for 7 minutes at 30°; then 0.1 ml of NADP (6.6 mg of free acid per ml, adjusted to pH 7.4) and 0.1 ml of glucose-6-phosphate dehydrogenase (0.322 units) were added. After 3 additional minutes, 0.1 ml of glucose-1-phosphate was added to facilitate the reaction. The final volume was 1.5 ml. The change in absorbance was followed at 340 mμ on a Cary 14 spectrophotometer.



(1) Galactose

(2) Diacetone galactose

(3) Diacetone galactose-6-phosphate

(4) Galactose-6-phosphate

(5) β-1,2,3,4-Tetraacetyl galactose-6-phosphate

(6) Galactose 1,6-diphosphate

Figure 1. Scheme for the Synthesis of Galactose 1, 6-Diphosphate

CHAPTER IV

RESULTS

Preparation and Properties of Lactose-1-Phosphate

Preparation of β-Lactose Octaacetate

Lactose and sodium acetate were dried *in vacuo* overnight at 70° C. β-lactose octaacetate was prepared according to Hudson and Johnson (35). 12.5 g of anhydrous sodium acetate were added to 200 ml of acetic anhydride at 100° C in a 2 liter flask with a reflux condenser. The mixture was heated nearly to boiling and 50 g of lactose (0.146 moles) were added slowly to the heated mixture over 5 minutes. A moderately vigorous reaction occurred while the lactose and sodium acetate dissolved in the mixture. After all lactose had dissolved, the solution was heated at 100° for an additional 10 or 20 minutes. The resulting mixture was poured into 4 liters of water at 4° C. The insoluble viscous residue was stirred and allowed to settle. Then the water layer was discarded and the residue was washed 3 times with cold water. After standing overnight in the cold, the residue solidified and was broken up with a glass rod, filtered on a Buchner funnel, washed well with water and dried *in vacuo* at 60° C. The product was ground in a mortar and extracted with warm ether by decantation until all ether-soluble material was removed. The residue was dried and dissolved in warm 95 % alcohol and upon cooling the product crystallized. The product was recrystallized from

alcohol 3 times and weighed 32.6 g (53 % yield). The observed melting point of this product was 141°. The theoretical yield is 55 % (35), and the literature melting point is 140-141° (6).

Phosphorylation of β-Lactose Octaacetate

2.2 g (5 mmoles) of lactose octaacetate and 3.92 g (40 mmoles) of phosphoric acid were separately dried *in vacuo* for several days in Thunberg tubes. The different arms of the Thunberg tubes were connected and further dried *in vacuo*. While under vacuum the phosphoric acid was heated in a water bath at 56° C until all the phosphoric acid was melted. β-lactose octaacetate in the side arm was slowly added to the melted phosphoric acid. This mixture was stirred by shaking the tube and the liberated acetic acid was trapped in a CO_2-acetone trap. The reaction mixture was then placed in a water bath at 56° C and after one and one-half hours the acetic acid evolution ceased. The brownish syrup was dissolved in 25 ml of tetrahydrofuran and the mixture was poured into 170 ml of ice-cold 1 N lithium hydroxide and allowed to stand overnight at room temperature to hydrolyze the acetyl groups (23). The white lithium phosphate residue was removed by filtration and the basic solution was passed through a column of Dowex 50W x 8 (2.3 x 30 cm) in H^+ form and the column was washed with 300 ml of water until the effluent of the column was at pH 4.5. The acidic effluent was made basic by addition of cyclohexylamine to pH 11. The resulting solution was then concentrated *in vacuo* to dryness. The residue was washed with absolute alcohol and then with 120 ml of dry isopropyl alcohol (dried over calcium sulfate). The mixture was centrifuged and the residue was washed twice with 25 ml portions of isopropyl alcohol. The product was

washed with dry ether (dried over sodium) and finally dried *in vacuo* over calcium chloride. The yield was 1.23 g (40 %, dicyclohexylammonium salt).

An attempt was made to crystallize lactose-1-phosphate as the potassium salt. The effluent from the Dowex 50W x 8 column was made basic by the addition of 0.5 N potassium hydroxide. This solution was concentrated *in vacuo* and a yellow-colored product was obtained which was dissolved in water and decolorized by treatment with activated carbon. However, upon concentration of the solution the yellow color appeared again and a crystalline product was not obtained. Treatment with ether or acetone, in place of absolute alcohol, did not produce a crystalline product.

The potassium salt of lactose-1-phosphate could be prepared from the cyclohexylammonium salt. In another preparation the yield of product as the cyclohexylammonium salt was 1.35 g (2.17 mmole, 45.5 % yield). The product, α-lactose-1-phosphate (cyclohexylammonium salt), was dissolved in 40 ml of water and passed through the Dowex 50W x 8 column (1.8 x 25 cm) in H^+ form. The strongly acid filtrate was made alkaline with 0.5 N potassium hydroxide solution to pH 8.5 and then concentrated under reduced pressure to 30 ml. Alcohol 95 % was added gradually to the ice-cold solution until a precipitate formed. The product was filtered and dried *in vacuo* overnight (yield 472 mg, 46 % conversion).

The product, lactose octaacetate, had a melting point of 141°, which is identical to that reported by Sasaki *et al.* (6). The infrared spectrum of lactose octaacetate is shown in Figure 2 which indicated that the product was β-anomer (40). The ratio of total phosphate to lactose (hydrolyzed by 1 N H_2SO_4 at 100° C for 20 min.) was 1.02:2.

The R_{Pi} values of lactose-1-phosphate and other related sugars are shown in Table I. The product contained no inorganic phosphates.

TABLE I

R_{Pi} VALUES OF LACTOSE-1-PHOSPHATE AND OTHER RELATED SUGARS*

Compounds	R_{Pi}
Inorganic phosphate	1.00
Glucose	1.35
Lactose	1.14
Galactose	1.33
Lactose-1-phosphate (Cyclohexylammonium salt)	0.76
Lactose-1-phosphate (Potassium salt)	0.76

*Solvent: 95 % ethanol : 1 N NH_3/Acetic acid (pH 3.5) 75 : 30 v/v; Whatman 1 MM; descending.

Infrared spectra of lactose octaacetate and lactose-1-phosphate are shown in Figure 2 and Figure 3, respectively.

Preparation and Properties of Galactose 1,6-Diphosphate

Preparation of Diacetone-D-Galactose

A mixture of 50 g galactose, 100 g of anhydrous copper sulfate, 100 ml of acetone, and 5 ml of concentrated sulfuric acid was stirred vigorously at room temperature for 30 hours. The mixture was filtered and neutralized with dry calcium hydroxide. The filtrate was concentrated under reduced pressure to a syrup and distilled carefully under

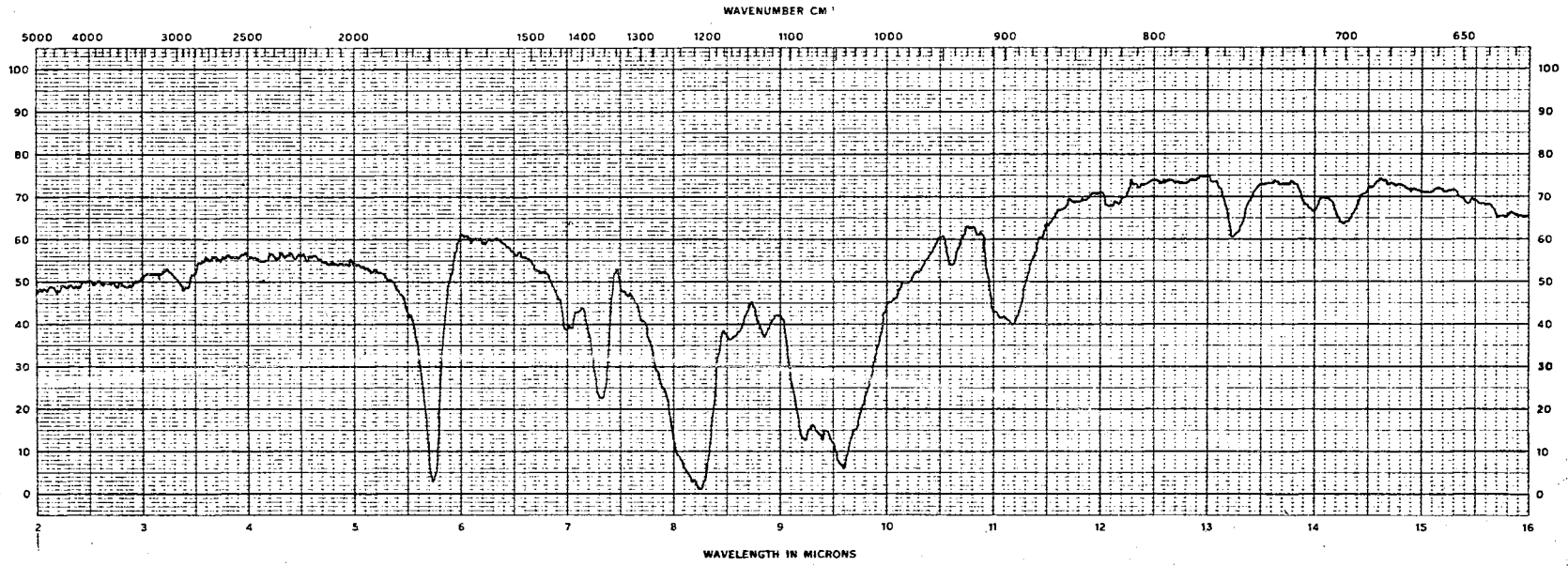


Figure 2. Infrared Spectrum of Lactose Octaacetate

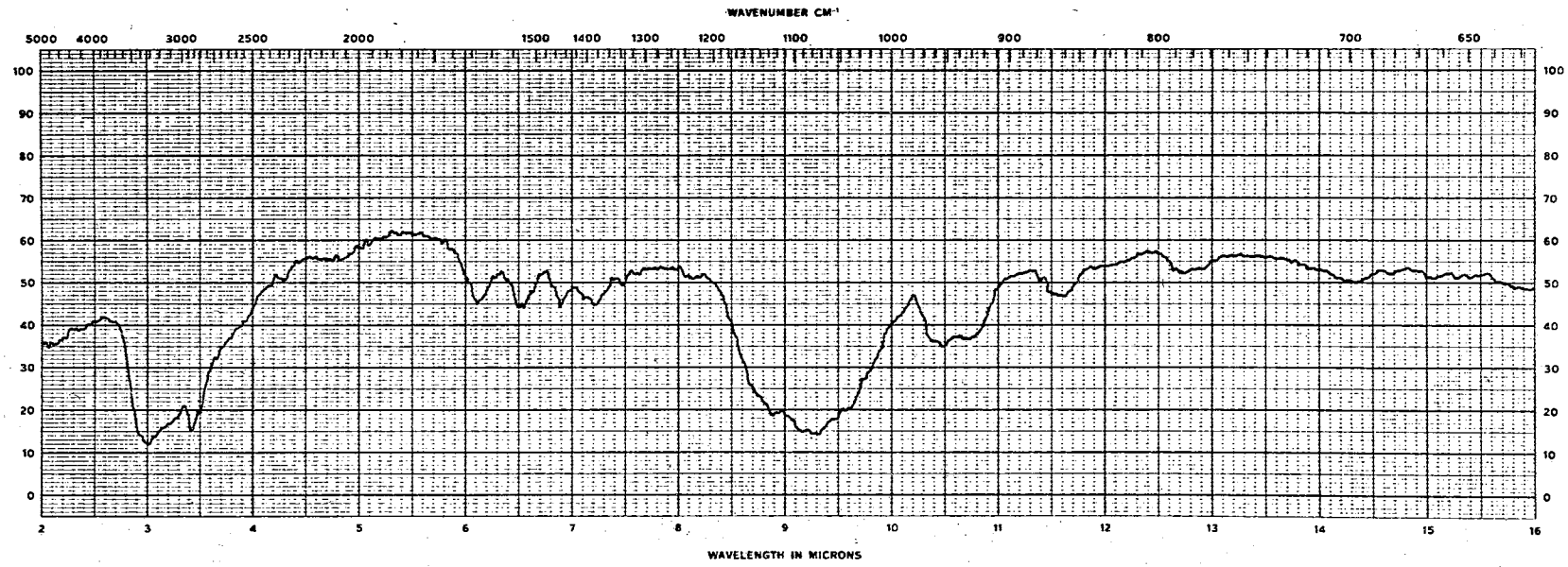


Figure 3. Infrared Spectrum of Lactose-1-Phosphate

reduced pressure. The yield of crude diacetone galactose, boiling at 131-135°, p = 0.2 mm (36) was 17.2 g.

Preparation of Galactose-6-Phosphate

Phosphorylation of diacetone-D-galactose was accomplished as follows: 6.5 ml (1.0 mole) of freshly redistilled phosphorous oxychloride were dissolved in 75 ml of dry pyridine and chilled to -30° in a 500 ml Erlenmeyer flask. A solution of 17.2 g (0.85 mole) of diacetone galactose in 50 ml of dry pyridine was added to the solution in four portions. The mixture was cooled to -30° before each addition and the temperature was not allowed to raise above -5°. Then a solution of 10 % water in pyridine was added slowly until no further heat was evolved. Additions of ice and ice-water were made to maintain the temperature below 10°. This mixture was neutralized with a warm saturated barium hydroxide solution (less than 50°), and the pyridine was removed by vacuum distillation in a 1000 ml 3-necked flask. Patience and care were needed in order to prevent any bumping during the course of the distillation. Additional water was added at intervals in order to assist in the removal of the pyridine and this was continued until only a faint odor of pyridine was evident. The acetone groups were hydrolyzed with 1 N sulfuric acid at 80° for 16 hours. Chloride ions were removed with freshly prepared silver carbonate; then the residual silver was removed as a precipitate of silver sulfate and the excess hydrogen sulfide was removed by aeration. A warm saturated barium hydroxide solution was added to the solution until the mixture became slightly alkaline and the residue was removed by centrifugation. The effluent was concentrated to 30 ml and the barium salt of α-galactose-6-phosphate was obtained by the

addition of an equal volume of 95 % alcohol. The crude product was washed with 50 % alcohol, redissolved and precipitated twice with 95 % alcohol. The purified product was dried over calcium chloride *in vacuo*. The yield was 5.2 g (20 %).

Preparation of 1,2,3,4-Tetraacetyl Galactose-6-Phosphate

In a test tube 1 ml of acetic anhydride (10 mmole) and 74 mg of freshly fused sodium acetate (0.9 mmole) were mixed and heated to boiling. 400 mg of galactose-6-phosphate (Ba salt, 1.5 mmole) was then gradually added in small portions. The mixture was shaken and heated occasionally. After the complete addition of galactose-6-phosphate, the brown-colored mixture was heated for 10 minutes, cooled and treated with 15 ml of absolute alcohol. The resulting mixture was heated slightly to decompose any excess acetic anhydride. The solution was evaporated on a rotary evaporator and a sticky residue was obtained. The residue was treated with 15 ml of dry ether and triturated until the residue solidified. The mixture was filtered and the residue was dried *in vacuo* over magnesium perchlorate. The yield was 561 mg. On subtracting the weight of sodium acetate (74 mg), the product weighed 487 mg, but theoretically the weight was 450 mg. The excess weight maybe due to moisture since the product is highly hydroscopic. The same phenomenon was observed by Khan *et al*. (33) in the synthesis of glucose 1,6-diphosphate.

Phosphorylation of β-1,2,3,4-Tetraacetyl Galactose-6-Phosphate

487 mg of crude 1,2,3,4-tetraacetyl galactose-6-phosphate (1.08 mmole) and 2.5 g (approx. 25 mmole) of anhydrous phosphoric acid were used in a slightly modified MacDonald's procedure (23,25). The ratio of

acetate to phosphoric acid was 1:12. Phosphorylation of the sugar was performed at 56° and in a similar manner to the procedure for lactose-1-phosphate. A column of Dowex 50W x 8 (1.8 x 25 cm) was used to acidify the basic solution after saponification with addition of lithium hydroxide. The acidic solution was concentrated *in vacuo*. Absolute alcohol was added and then removed *in vacuo* and this procedure was repeated several times. Finally a yellow residue was obtained which was washed with 100-150 ml of dry isopropyl alcohol (dried over calcium sulfate). The precipitate was filtered and washed with dry ether and dried *in vacuo* over calcium chloride. The yield was 204 mg (0.285 mmoles).

Inorganic phosphoric acid in the crude product of galactose 1,6-diphosphate was removed by dissolving the product in 30 ml of water and adding a solution of 70 mg of magnesium acetate in 30 ml of 14 % ammonium hydroxide to form the insoluble magnesium ammonium phosphate. Cations were removed by a small column of Dowex 50W x 8 (2.2 x 20 cm) and the percolate was made basic with cyclohexylamine and concentrated *in vacuo* to a syrup which was dissolved in 10 ml of 95 % ethanol and held in the cold. The product was filtered and dried *in vacuo* over magnesium perchlorate. 168 mg (0.236 mmole) of galactose 1,6-diphosphate were obtained.

The ratio of total phosphate to acid labile phosphate was 1.8:1. However no evidence of reducing sugar was found in the final product. This indicated that no galactose-6-phosphate was present in the product. The R_{Pi} value from ascending paper chromatography (solvent: methanol-formic acid-water 80:15:5 v/v, containing 0.2 gm EDTA/100 ml) on Whatman 3 MM was 1.02 compared to inorganic phosphate as 1.0. Also, no evidence of inorganic phosphate was found in the final product. The infrared

spectrum for galactose 1,6-diphosphate is presented in Figure 4.

The inhibition of galactose 1,6-diphosphate on the phosphoglucomutase reaction was determined by the coupled assay method of Ray et al. (41). It was necessary to use glucose-1-phosphate free of glucose 1,6-diphosphate (Sigma Grade V) since the inhibition was not observed with glucose-1-phosphate (Type I) which contains a small amount of glucose 1, 6-diphosphate. It was found that galactose 1,6-diphosphate contained a very small amount of glucose 1,6-diphosphate as there was a slight reaction rate when the phosphoglucomutase reaction was measured with galactose 1,6-diphosphate as the substrate in place of glucose 1,6-diphosphate. It further was determined that the ratio of product galactose 1,6-diphosphate to glucose 1,6-diphosphate was 3500:1. This result would indicate that the starting material, galactose, contained a small amount of glucose which was not detectable by the usual chormatographic methods.

The reaction rate of phosphoglucomutase with the substrate glucose 1,6-diphosphate, and, glucose 1,6-diphosphate and galactose 1,6-diphosphate was replotted as 1/V versus 1/S in Lineweaver-Burk plot for determining the K_m for glucose 1,6-diphosphate which was 2.63×10^{-8} M (Figure 5). Galactose 1,6-diphosphate was a competitive inhibitor with respect to glucose 1,6-diphosphate.

The K_i for the galactose 1,6-diphosphate was determined as 0.34 mM from the plot of the slope versus the inhibitor galactose 1,6-diphosphate (Figure 5). Galactose 1,6-diphosphate inhibited the phosphoglucomutase reaction in which glucose 1,6-diphosphate is required. However the degree of inhibition by galactose 1,6-diphosphate (Figure 6) was much less than that of the stimulation by glucose 1,6-diphosphate. This

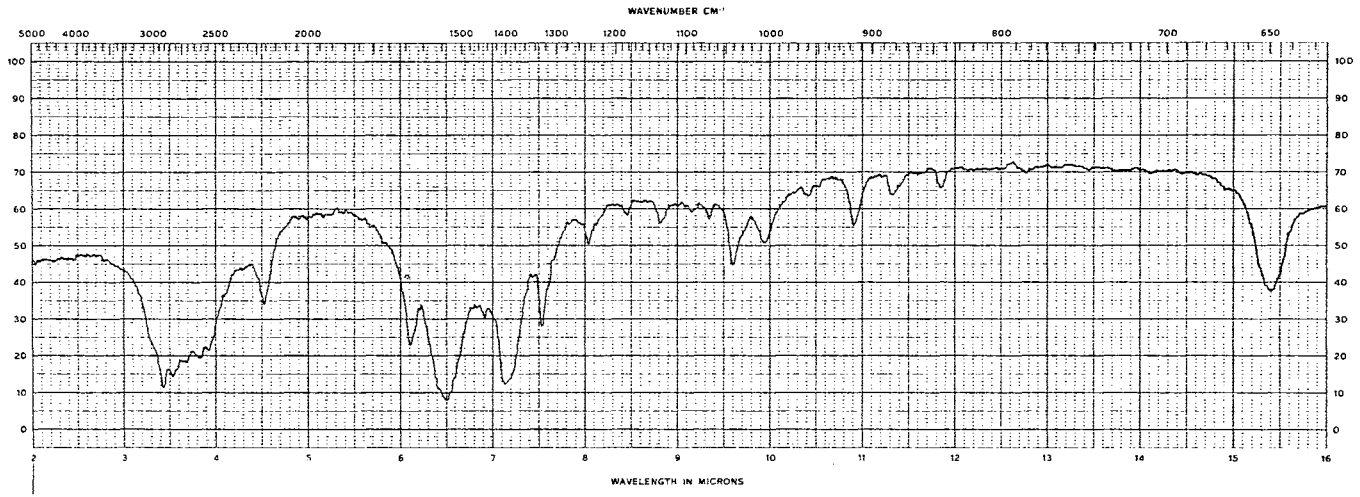


Figure 4. Infrared Spectrum of Galactose 1,6-Diphosphate

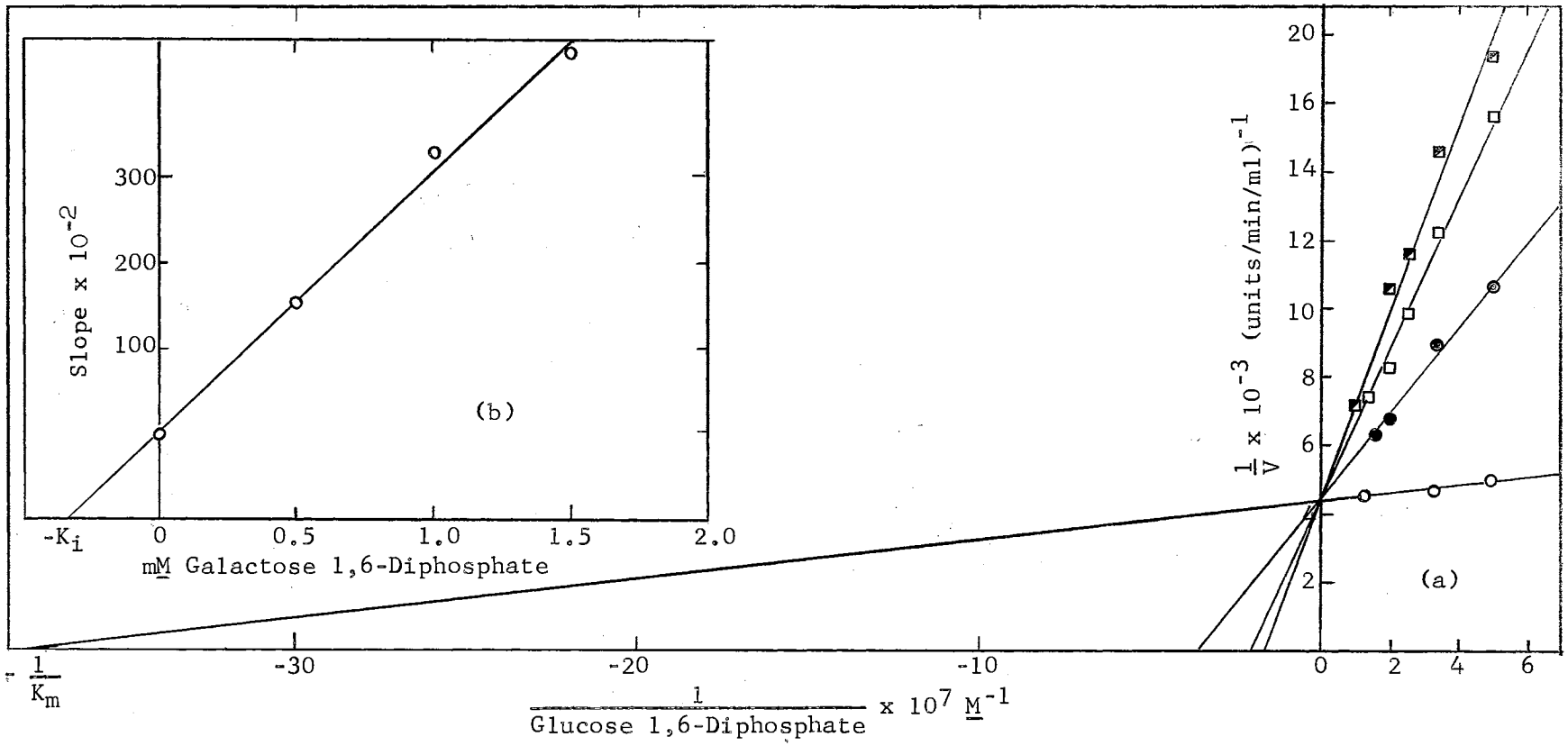


Figure 5. Lineweaver-Burk Plot for Determining the K_m of Glucose 1,6-Diphosphate and the Plot for Determining the K_i of the inhibitor galactose 1,6-Diphosphate.

(a) Double reciprocal plot of velocity versus glucose 1,6-diphosphate. O-O was glucose 1,6-diphosphate; ●-●, 0.5 mM galactose 1,6-diphosphate; □-□, 1.0 mM galactose 1,6-diphosphate; ▨-▨, 1.5 mM galactose 1,6-diphosphate. Galactose 1,6-diphosphate was added prior to the addition of substrate, cofactor, glucose 1,6-diphosphate to start the reaction. The rate observed with the galactose 1,6-diphosphate was subtracted from the rate with presence of glucose 1,6-diphosphate to correct for the small amount of glucose 1,6-diphosphate in the synthesized product. (b) A secondary plot of the slope versus galactose 1,6-diphosphate from the data in (a). The K_i for galactose 1,6-diphosphate was 0.34 mM.

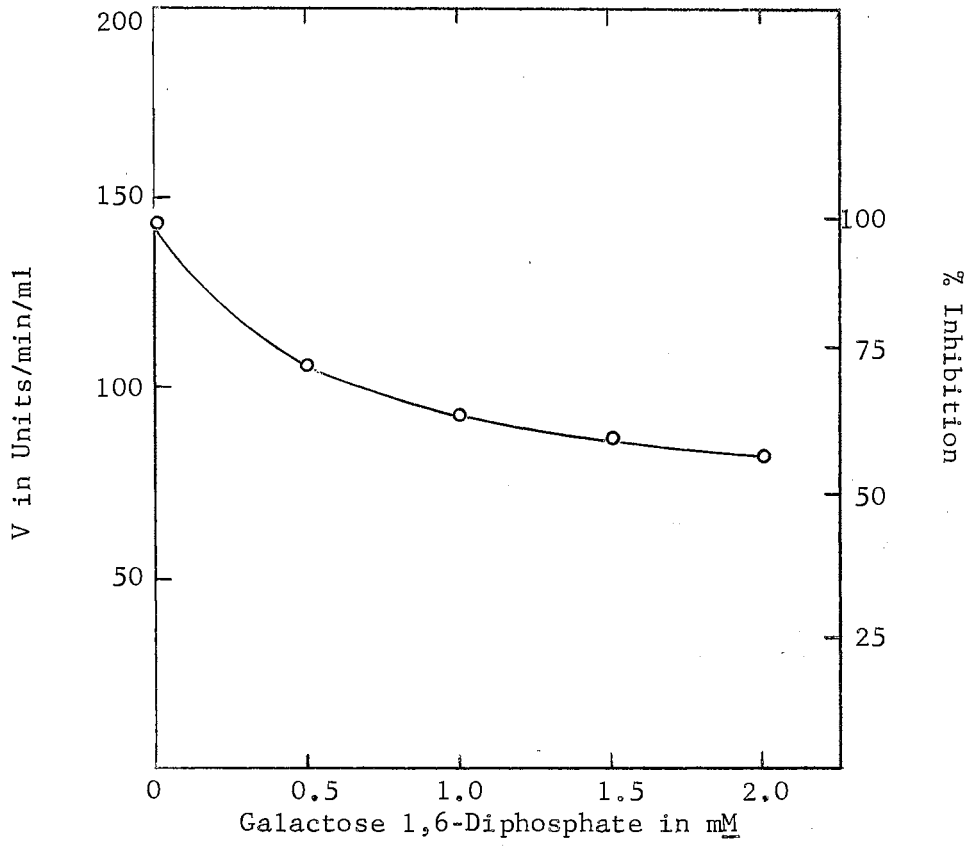


Figure 6. Effect of Galactose 1,6-Diphosphate on the Phosphoglucomutase Reaction

Varying amounts of galactose 1,6-diphosphate were added to the reaction mixture of phosphoglucomutase after the addition of 2×10^{-5} M glucose 1, 6-diphosphate. The percentage of inhibition is also shown in this plot.

indicated that galactose 1,6-diphosphate was a poor inhibitor although it was competitive.

CHAPTER V

DISCUSSION

The purpose of this investigation was to apply the MacDonald method (23) to the synthesis of α-lactose-1-phosphate and the galactose 1,6-diphosphate.

Comparison of the infrared spectrum with literature values (8.5-9.0 mμ, 6.4 mμ, and 10.7 mμ) for lactose octaacetate (40) confirmed that the product obtained in this experiment was the β-anomer. This result would suggest that the phosphorylated sugar is the α-anomer of the lactose-1-phosphate as the phosphorylation of β-lactose octaacetate would be expected to give inversion. The final product was tentatively assumed as the α-anomer of the sugar phosphate.

Gander, Peterson, and Boyer (3,42) reported on the synthesis of lactose-1-phosphate from UDPGlu and glucose-1-phosphate by enzymatic fractions from bovine mammary tissue. However they presented no evidence to show the presence of an enzyme which could hydrolyze lactose-1-phosphate to the lactose and inorganic phosphate in the mammary gland. In 1961, Watkins and Hassid (43) presented a convincing and reasonable pathway for the biosynthesis of lactose. They proposed that the final stage in the formation of lactose involves the transfer of D-galactose from UDP-D-galactose to D-glucose according to the reaction,

$$\text{UDPgalactose} + \text{Glucose} \xrightarrow{\text{Galactosyl transferase}} \text{Lactose} + \text{UDP} \quad .$$

To date, no other laboratory has confirmed the work of Gander, Peterson and Boyer (3,42), and at the present time it is questionable if lactose-1-phosphate has any biological function.

Posternak (9) suggested that sugar 1,6-diphosphates may be synthesized by the introduction of a second phosphate group into suitable derivatives of either (a) sugar-6-phosphate or (b) sugar-1-phosphate. He reported that the former method was more successful than the latter. In this investigation, galactose 1,6-diphosphate was synthesized by the introduction of a second phosphate group into the 1,2,3,4-tetraacetyl galactose-6-phosphate.

MacDonald (25) reported the results of some experiments in which inversion did or did no occur, depending on the kinds of sugar acetate used. Acetylated sugars with the 1,2-trans diequatorial configuration reacted very well in the phosphorylation reaction while the 1,2-cis configuration made difficult the displacement of acetate on the C-1 position.

The MacDonald procedure (23) has been widely and successfully adopted in the phosphorylation of various sugars and again in these experiments, this relatively simple technique produced the desired sugar phosphates.

Metabolic and biological functions and properties of α-lactose-1-phosphate and α-galactose 1,6-diphosphate still remain unknown. Based on paper chromatography, infrared spectra and hydrolysis characterizations, the synthesized compounds were identified as α-lactose-1-phosphate and galactose 1,6-diphosphate.

The inhibition of galactose 1,6-diphosphate on the phosphoglucomutase reaction was determined. It was found that galactose 1,6-

diphosphate had a K_i of 0.34 mM and showed competitive inhibition with respect to glucose 1,6-diphosphate in the phosphoglucomutase reaction.

A slight reaction rate was observed in the phosphoglucomutase reaction when galactose 1,6-diphosphate was used as the substrate. This suggested that the starting material galactose contained a small amount of the glucose, which later was converted to glucose 1,6-diphosphate in the course of the synthesis of galactose 1,6-diphosphate. The ratio of the phosphoglucomutase reaction was corrected by subtracting initial rate exhibited in the presence of galactose 1,6-diphosphate.

CHAPTER VI

SUMMARY

The phosphorylation of β-lactose octaacetate with fused anhydrous phosphoric acid at 56° C for two hours subsequently gave purified α-lactose-1-phosphate. Descending paper chromatography showed a R_{Pi} value of 0.76 and this was compared to other related sugars. The ratio of reducing sugar (after hydrolysis) and 7-min phosphate was 2:1.02. Evaluation of infrared spectrum led to the conclusion that β-lactose octaacetate was obtained. It was concluded that the final product was α-lactose-1-phosphate. The yield of α-lactose-1-phosphate was 45.5 % based on lactose octaacetate as a starting material.

Galactose-6-phosphate was obtained from diacetone galactose by the procedure described by Levene and Meyer (36). Acetylation of galactose-6-phosphate yielded 1,2,3,4-tetraacetyl galactose-6-phosphate which was then phosphorylated by the MacDonald method. The yield for the final product α-D-galactose 1,6-diphosphate was 19.0 %. The product contained acid labile and total phosphate (acid stable and acid labile) in 1:1.8 ratio. A representative infrared spectrum was shown. It was concluded that the product was α-D-galactose 1,6-diphosphate. Evidence for the inhibition of phosphoglucomutase from rabbit muscle by α-D-galactose 1, 6-diphosphate was found. The experimental results suggest that galactose 1,6-diphosphate was a competitive inhibitor to glucose 1,6-diphosphate in the phosphoglucomutase reaction.

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PART II

INDUCTION OF TYROSINE TRANSAMINASE IN ISOLATED RAT LIVER CELLS

CHAPTER I

INTRODUCTION

There is little doubt that the living cell is the fundamental unit of all living organisms. Yet, there is no such thing as a typical cell. The cells of different tissues are as different in morphology as they are in function. Currently the cell biologist seeks to explain in molecular terms what he observes with the aid of his instruments whereas the biochemist is interested in the structure of the cell and in the biochemical activity in which it is engaged.

The goal of this study was to attempt to induce tyrosine transaminase (EC 2.6.1.5.) with a synthetic corticoid in isolated, intact parenchymal cells from rat liver. The system of isolated, intact cells would be most convenient for studying hormone action in an *in vitro* system under controlled conditions. In mammals, one of the major means of controlling biochemical pathways is by way of hormone action and in a sense hormones may be considered as chemical regulators. There is evidence to suggest that hormones may exert their actions at the membrane level or the transcription and translational levels of protein synthesis. Certain types of cancer, especially mammary and prostate, are susceptible to hormonal therapy though the underlying reason for this action is not understood. It is clear that there is more than a casual relationship between hormones and cancer and for this reason systems readily amenable to experimentation become an important facet in studying the etiology

of hormone action and caner control by hormones. For example, the endocrine glands and their respective hormones directly cause tumors (1). Several strains of mice exist with a relatively high natural incidence of mammary cancer; if their ovaries are removed prior to the development of mammary tumors the number of such tumors is reduced. The function of the ovaries is to control mammary development by the secretion of hormones, and in their absence the mammary glands do not grow.

It is true that very little is known as yet about the action of hormones on cells in vitro. Cells cultivated under in vitro conditions would seem to provide an ideal tool for the study of hormone action. It seems quite certain that the presence of hormones is not essential for cell survival but in view of their great importance in the intact animal, it is likely that they are required for the full development and function of differentiated cells.

The study of hormone action in whole animals is difficult since the effects observed upon hormonal administration may arise from a series of complex biochemical events. Investigators are searching for simpler systems in order to study the primary effect of hormones. In theory, isolated cells are easier to study than tissues or whole organisms, provided they show the same response to the hormone as is observed in slices and whole organism studies. In the liver, cortisol induces the formation of certain specific enzymes which may be used as markers for studying hormonal induction in isolated rat liver cells. Previously isolated rat liver cells (2) did not respond to hormones but it appears that a new isolation procedure may produce cells that would be responsive to hormones.

The present study is an attempt to induce tyrosine α-ketoglutarate

transaminase in isolated parenchymal rat liver cells by treatment of dexamethasone phosphate (Dex), a synthetic glucocorticoid.

CHAPTER II

LITERATURE REVIEW

Preparation of Isolated Liver Cells

Prior to 1953, liver cells were prepared in low yields by forcing liver through cheesecloth (3), by shaking tissue with glass beads (4,5), by mincing with a tissue press (6), or by homogenization with a Potter-Elvehjem grinder (7).

Later, considerable use was made of chelating agents as a means of dispersing animal tissue cells. Anderson (8) dispersed rat liver cells with various chelating agents including sodium citrate and Versene (ethylenediaminetetraacetic acid; distributed by Versenes, Inc., Framingham, Mass.). Other methods of dispersing liver cells also included filtration through a stainless steel sieve (9), incubation in acid solution (10), and use of enzymatic (tryptic) digestion (11,12).

In 1954, Zwilling (13) reported the use of Versene, together with calcium-magnesium-free saline, for dissociating cells from chick embryos. Rinaldini (14) suggested that more information on the permeability of the cell membrane to these substances was desirable before they are used routinely in culture work. Rinaldini (14) found that citrate, Versene, and glycine, in concentrations of 0.1 and 1.0 per cent were much less effective than proteolytic enzyme for disintegrating minced embryonic heart tissue. Liver chelating agents, unlike proteolytic enzymes are

not inhibited by serum and unless they are washed away they may do lasting damage to the cells (14).

The work of Castagna and Chauveau in France (15) suggested utilization of Ficoll, a synthetic polysaccaharide which was first introduced by Holter and Mollder (16), to preserve the integrity of the cell suspension. They also found that various concentrations of Ficoll did not damage the cells because of its low osmotic effect. Although they claimed the isolated cells were morphologically intact, the extensive application of mechanical methods in the course of dispersion will greatly affect the integrity of the isolated cells.

Rappaport et al. (17) prepared cell suspensions from mouse liver in 1966 by using sodium tetraphenylboron (TPB). TPB is a specific agent for complexing potassium ion (K^+). They suggested that the dissociation of liver tissue is the result of the removal of K^+, which is the major cation involved in aggregation of the cells in this tissue. Furthermore, they suggested the cells were held together predominantly in the tissue by coordination through K^+. Their interesting results on different tissues (18) suggest that coordination through monovalent cation is a general mechanism for aggregation of cells to form tissues. They also reported that higher temperatures facilitated dissociation. The efficiency of dissociation is largely influenced by the TPB concentration, temperature and pH. The quality of the cells released both in the presence of TPB and in the sucrose salt control was greatly improved when the temperature was increased. Also the cells released were larger and less granulated than those obtained at 4° C.

The findings of Rappaport and her coworkers (17,18) indicated that the most efficient dissociation of a given tissue will be affected by

the use of the appropriate monovalent cation complexing agent under conditions supporting metabolism of the cells and destabilization of any intracellular material. Accordingly, they proposed that the cells released by TPB retain the gross morphology of the various cell type *in situ*. Unfortunately they did not determine whether these isolated cells are suitable for any biochemical experimental use.

Yamada and Amborose (19) prepared cell suspension from mammalian cells by using a collagenase and hyaluronidase treatment. They reported that the cell suspension prepared by this method had little effect on the measurement of electrical charge of surface membranes.

In 1967, Howard *et al*. (20) reported a method for the preparation of isolated parenchymal cells from rat liver. Rat liver was incubated with 0.15 % collagenase and with 0.15 % hyaluronidase to yield adequate numbers of cells for biological experimental purposes. Previous methods have resulted in cells with damaged membranes and as a consequence the soluble compounds of the cells were lost to the medium. Such cells failed to respond to a hormonal treatment which would induce certain enzymes under *in situ* conditions (2).

The cells prepared by the procedure of Howard *et al*. (21) had a high endogenous respiration rate and were not stained by a vital stain suggesting that these cells had a normal membrane. Recent experiments by Tompkins *et al*. (22), with a minimum deviation hepatoma, have shown that such cells are induced to form tyrosine transaminase by cortisol. It would appear that isolated liver cells, providing their cell membranes were intact, should also form tyrosine transaminase in the presence of other glucocorticoids.

In a later report by Howard *et al*. (21), a modification of the

original procedure was described. In the new procedure, an enzyme solution of 0.05 % collagenase and 0.10 % hyaluronidase is used and this is followed by a minimum mechanical treatment. Accordingly, the yield of cells is less than the previous method, but most of the cells (90 to 95 %) appear structurally intact and the viability of the cells varies from 75 to 95 % in different preparations. They report that these cells will maintain linear rates of respiration for periods of two hours, and measurable oxygen uptake can be maintained for as long as three days. Their results confirmed the results obtained in our laboratory, that decreased amount of enzymes greatly improved the integrity of the cells.

Preparation of Isolated Cells From Other Sources

In 1916, Rous and Jones (23) treated tissue cultures with trypsin for one hour at 37^{o} C and were able to prepare subcultures from the dispersed cells. Good results were obtained both from avain and mammalian tissues, including spleen, connective tissue, endothelium, and malignant tumors, but epithelial sheets were not readily dispersed.

In 1941, Medawar (24) used trypsin to obtain viable cells from chick heart cultures and to separate the epidermal layer of human skin from the dermis. In 1952, Moscona (25) reported cultivation experiments in which the cells of embryonic rudiments were dispersed with trypsin in a saline solution without calcium and magnesium. Since the beginning of the century (26) these divalent cations were known to promote the stability of the intercellular matrix. In 1943, Chambers and Cameron (27) succeeded in separating epithelial cells in cultures by treating them with calcium-free saline. However, Zeidman (28) found that the lack of both calcium and magnesium proved more effective in lowering adhesiveness

than the lack of either cation alone. In 1952, Dulbecco (29) introduced the trypsin procedure to virology by demonstrating macroscopic virus lesions in cultures of chick-embryo cells prepared with the aid of trypsin.

Extensive use of trypsin in the preparation of cell suspensions was investigated in various laboratories. The most efficient trypsinizing procedures were devised for monkey-kidney tissues, as a means of obtaining maximal yields of viable cells for use in polio vaccine programs.

In 1954, Dulbecco and Vogt (30) used trypsin for the preparation of cell suspensions from monkey-kidney tissue. The procedure of Dulbecco and Vogt was soon modified by Younger (31). Soon both methods were refined by Rappaport (32) who developed a more efficient procedure which allowed for a continuous renewal of trypsin and the continuous withdrawal of the cell suspension. Madin and his associates (33) prepared swine-kidney cells by agitating a kidney-trypsin mixture. Cooper (34) prepared suspension of whole chick embryo by forcing embryos through a syringe and digesting the resulting pulp with trypsin (35), a procedure now widely used for obtaining relatively small quantities of tissue.

It soon became obvious that trypsin-dispersed cells are unusually fragile and should be removed from the trypsinizing chamber as soon as they are released from the tissues so as to protect them from mechanical damage or excessive digestion with trypsin.

Medearis and Kibrick (36) also prepared trypsin-dispersed cell suspensions from mouse embryos and the brains of new born mice.

Rinaldini (37) has studied the effect of several enzymes including trypsin, pancreatin, elastase, and papain, on various chick tissues. He found that trypsin preparation was found to vary largely in their ability

to disintegrate a given tissue; crude pancreatin was more damaging to cells than trypsin; crude elastase is capable not only of hydrolyzing the mucoid residue but also of producing by itself discretely isolated cells in high yield from chick-embryo heart, breast muscle, liver, and unkeratinized skin.

Lasfargues (38) and Hinz and Syverton (39) have used collagenase successfully in the dispersions of cells from organs rich in connective tissue. Lasfargues used collagenase to separate ducts and acinic of the mammary gland of mice from the adipose tissue surrounding them. Hinz and Syverton (39) used commercially prepared collagenase (Worthington Biochemical Corp., Freehold, N. J.) in the preparation of cell cultures from human, swine, and rabbit-lung tissue.

It is clear that several enzymes are useful for dispersing tissue into dispersed cells. At this time it is difficult to tell whether the use of a particular enzyme as a dispersing agent affects the nature of the intracellular material since most of the enzymes used are impure and it may be that a contaminant enzyme would give a better insight into the natures of cellular adhesion in tissues.

Metabolism of Dispersed Rat Liver Cells

The use of dispersed rat liver cells for metabolic studies has attracted many investigators. Although the disruption of the cell and the study of metabolic reactions in different subcellular fractions has provided insight on the organization of function within the cell, few papers are available on enzyme leakage from intact liver cells in suspension.

In earlier studies, satisfactory preparations of intact, dispersed

cells were difficult to achieve, and organ slices or whole organs were the most satisfactory form for the study of intact cells. Earlier preparations of intact, dispersed cells were not suitable for biochemical studies due to the damage of the cell in the dispersion process. These cells were characterized by a low rate of respiration and enzyme leakage.

In 1962, Berry (40) demonstrated that the metabolic activities of dispersed mouse liver cells were critically dependent on the composition of suspending medium. Endogenous respiration was greatest when the sucrose concentration of the medium was 0.1 M or lower, and was nil in 0.3 M sucrose medium. These isolated mouse liver cells oxidized citric acid cycle intermediates, glutamate, lactate, pyruvate, β-hydroxybutyrate, α-glycerophosphate and fatty acids. Cells incubated in Kreb's phosphate-saline did not respire. Berry (40) proposed that the lack of respiration in the medium was related to the increased permeability of the cell membrane with penetration of calcium ions and orthophosphate (41) into the cells causing mitochondrial swelling and destruction.

Henley et al. (42) showed that enzymes such as glutamic-pyruvic and glutamic-oxaloacetic transaminases, and lactic dehydrogenase, easily leaked out from suspensions of rat liver.

Takeda et al. (43) were among those who have been working to obtain a satisfactory preparation of dispersed rat liver cells. However, tests of enzyme activity showed a large loss of various enzymes, particularly those in the soluble fraction of the cell. The enzymes that were almost completely lost included serine and threonine dehydrogenase, tryptophan pyrrolase and lactate dehydrogenase. However, microsomal enzymes such as a glucose-6-phosphatase and mitochondrial enzymes of the citric acid cycle were retained in the cell.

The failure to utilize glycolytic intermediates may be due to the disruption of the plasma membrane with loss of protein, neutral lipids and certain soluble enzymes such as adolase, malate dehydrogenase, lactate dehydrogenase and L-iditoldehydrogenase (44).

Takeda et al. (43,45,46,47) have made extensive use of dispersed rat liver cells for the elucidation of the mechanisms regulating metabolism and the studies of the biochemistry of the animal cells. Ichihara et al. (45) found that cellular respiration was regulated by oxidative phosphorylation in the dispersed rat liver cells. This was shown by the stimulation of cellular respiration by ATP. The response of the rat liver cells to glucocorticoids such as cortisone, cortisol, and dexamethasone (16 α-methyl-9-α-fluro-1-dehydrocortisol) was studied by Takeda et al. (43). Their actions on enzyme leakage were parallel with their anti-inflammatory potencies. Dexamethasone increased glutamic-pyruvic transaminase activity in the soluble, but not in the mitochondrial fraction of rat liver (43).

The interrelation between nucleic acid and protein formation in dispersed rat liver cells was investigated by Inoue et al. (46). They confirmed that the rate of protein and RNA synthesis in dispersed cells rose linearly during the first 2 hours of incubation, whereas those of a homogenate decreased with time reaching a steady level after 30 minutes. Inoue et al. (46) also observed that puromycin strongly inhibited amino acid incorporation of uridine into protein but not RNA synthesis, and actinomycin was less inhibiting for protein synthesis when not preincubated with the cells. Their results supported the idea that protein synthesis in dispersed cells is controlled by the formation of messenger RNA. Again, Ichihara et al. (45) subsequently investigated lipid

synthesis in the dispersed rat liver cell suspensions. The dispersed cells showed considerable incorporation of acetate into lipids. With regard to a hormonal effect on lipid synthesis, epinephrine and dexamethasone inhibited synthesis, while insulin stimulated it only in the presence of pyruvate. A detailed report on the action of insulin on lipid synthesis in dispersed cells was reported by Adachi et al. (47). They also presented evidence that insulin stimulates the carboxylation of acetyl CoA to form malonyl CoA in the dispersed liver cells.

Recently Howard and Pesch (21) reported that cells prepared by the Howard et al. (20) procedures had high respiratory rates in Krebs-Ringer phosphate solution and in glucose-free Hanks solution which suggested that these cells are structurally intact.

The responsiveness of the dispersed cells to the addition of hormones indicate that cell suspension may provide a suitable system for studying hormonal action on cellular metabolism.

Induction of Tyrosine Transaminase

The regulation of metabolism by the induction and repression of specific enzymes is well established in bacteria, where substrates or products of the affected enzymes are often the effector agents. Although similar controls exist in animal cells (48), the regulation of metabolism in higher organisms is apparently more complicated and other effector agents, such as hormones, are involved in this regulation.

The induction of several hepatic gluconeogenic enzymes by glucocorticoid hormones (49) has been studied as a model of hormonal control of protein synthesis in mammalian cells. Other researchers also have demonstrated that cortisol-stimulated induction of tyrosine transaminase

in rats is associated with rather striking increases in total hepatic RNA.

In 1964, Pitot et al. (50) reported briefly that the Reuber hepatoma in tissue culture showed an increase in tyrosine transaminase activity in response to the addition of hydrocortisone.

In studies with the Reuber H-35 hepatoma, Pitot et al. (51) were able to show that the basal level of tyrosine transaminase in the tumor grown in vitro was like that of tumor grown in vivo in the adrenalectomized rat. Thompson et al. (52) reported in 1966 that a rapid, substantial increase in the activity of tyrosine α-ketoglutarate transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5.) was observed by addition of glucocorticoids to the cell line (designated HTC for hepatoma tissue culture), which is a newly established line of tissue culture cells derived from a primary culture of the ascites form of an experimental rat hepatoma.

Recently, it was shown in another cell line, Reuber H-35 hepatoma, that tyrosine transaminase could be induced by hydrocortisone, insulin, and glucagon (53).

In a more recent study, Kenny and Hager (54) observed that hydrocortisone, insulin, and glucagon were all found to induce tyrosine transaminase in the isolated perfused rat liver. The response to each of these hormones was essentially identical with that observed in experiments in vivo. However, growth hormone did not alter tyrosine transaminase activity in this system (54). Granner et al. (55) reported that glucocorticoids induce TAT (tyrosine aminotransferase activity in rat liver and HTC cells. They concluded that TAT induction is an early effect of glucocorticoids and TAT induction in HTC cells as well as in

liver is due to an increased rate of synthesis of the enzyme. Moreover, the induced rate of synthesis of TAT depends on the constant presence of hormone.

Work with the 5123 hepatoma (56) showed that the tumor exhibited very high levels of this enzyme in the intact host. Tyrosine administration increased the tumor enzyme only slightly while bringing the host liver tyrosine transaminase to tumor levels. Cortisone administration almost doubled the tumor basal level while giving the normal response in the host liver (57). Adrenalectomy of the host lowered the tumor enzyme to near that of liver, and cortisone administration caused a response in both the liver and tumor of the adrenalectomized tumor-bearing host. In contrast to the low response of tryptophan pyrrolase to cortisone administration, tyrosine transaminase appear to be "overresponsive" to the presence of adrenal steroids (57).

Tyrosine α-ketoglutarate transaminase shows a circadian rhythm in the intact rat as reported by Civen et al. (58). They reported that adrenal hormones do not play a major role in determining the circadian rhythmical pattern of TKT. Their studies suggest that the sensitivity of an enzyme's regulating system to inducing agents may be related to the inherent circadian rhythm of the enzyme (58).

Insulin and glucagon also bring about an induction of rat liver tyrosine α-ketoglutarate transaminase in vivo (59) in the isolated perfused liver (60) and in organ cultures of fetal rat liver (61). Induction of tyrosine α-ketoglutarate transaminase by hydrocortisone is more extensive than that obtained with the protein hormones. Wick (62) reported that epinephrine and the N^6-$O^{2'}$-dibutyryl analog of adenosine-3', 5'-cyclic phosphate both are effective inducers of tyrosine

α-ketoglutarate transaminase in explants of fetal rat liver maintained in organ culture. Wick (62) suggested that cyclic adenylic acid is an intermediate in the induction by epinephrine (59). Hydrocortisone increases the synthesis of tyrosine α-ketoglutarate transaminase whereas epinephrine induces this enzyme by a mechanism involving cyclic AMP (62).

Histological and biochemical studies supported the conclusion that hepatocytes remained healthy over 3 days in culture. The induced level of the transaminase was maintained unless steroid was removed, and then the activity fell rapidly back toward the control level. Only glucocorticoids and the protein hormones insulin and glucagon were active inducers among a number of hormones tested. Cycloheximide and actinomycin D prevented enzyme induction, but hydrocortisone did not affect either general RNA or protein synthesis (61).

Wogan and Friedman (63) reported that aflatoxin B_1 is a potent inhibitor of hydrocortisone-induced increases in rat liver tyrosine transaminase and tryptophan pyrrolase. Because of the similarities of effects of aflatoxin B_1 and of actinomycin D, they suggested that both inhibitors may have similar mechanisms, namely, supression of DNA-dependent RNA synthesis.

Very recently Kröger *et al.* (64) in Sweden observed that L-tryptophan increased tyrosine transaminase threefold with respect to non-induced controls and more than twice after induction with tyrosine plus cortisone acetate.

The antibiotic cycloheximide, an inhibitor of protein synthesis, has been used to block the degradation of tyrosine transaminase *in vivo* (65). However, in perfused liver, only the synthesis of tyrosine transaminase is inhibited, and no effect on degradation can be measured (66).

Therefore, Seglen (67) suggested that the effect of cycloheximide on degradation *in vivo* is an indirect one.

The bulk of the available experimental evidence shows that tyrosine transaminase is an enzyme readily induced by a variety of hormones in liver cells existing in different environments. The fact that the enzyme may be induced in minimum deviation tumor cells suggests that the hormones are effective at the cellular level and that the enzyme should be induced in isolated liver cells, provided they may be isolated with intact membranes.

CHAPTER III

EXPERIMENTAL PROCEDURES

Materials

Albino rats, obtained from the Holtzman Rat Co., Madison, Wis., weighing 100-200 g were used throughout. Collagenase was obtained from Schwarz Bioresearch, Inc., Orangeburg, N. Y.. Hyaluronidase (Type I), α-ketoglutaric acid, ρ-hydroxyphenyl pyruvic acid and cycloheximide were obtained from Sigma Chemical Co.. Pyridoxal-5'-phosphate (Codecarboxylase) and actinomycin D were from Mann Research Laboratory, New York. Eosin Y was from Allied Chemical Corp., N. Y., and Trypan blue was obtained from K & K Laboratories, Inc., Plainview, N. Y..

Methods

ρ-Hydroxyphenyl pyruvate tautomerase (EC 5.3.2.1., phenylpyruvate ketoenol-isomerase) was prepared from beef liver by a slightly modified procedure (68,69). All glassware was washed with Alconox (Alconox Inc., New York) and siliconized. The enzymes were dissolved in calcium-free Hanks solution (70). The procedure for isolation of rat liver cells was principally the one devised by Howard et al. (20,21).

Assay Method

Tyrosine transaminase was assayed as follows: liver cells were

disrupted in a Sorvall Omni-mixer (Ivan Sorvall, Inc., Norwalk, Conn.) at 10,000 rpm and the broken cell suspensions were centrifuged at full speed in an International clinical centrifuge. The supernatant solution was assayed for tyrosine α-ketoglutarate transaminase activity by the following modification of the method of Lin and Knox (71). A total volume of 1.0 ml contained a final concentration of 0.5 M sodium borate, 0-0.2 ml of the centrifuged cell supernatant solution in the Hanks buffer (a phosphate buffer), 3×10^{-3} mM of tyrosine, 0.05 ml ρ-hydroxyphenyl pyruvate-enol-keto tautomerase (3.4 units/ml), 50 μg of pyridoxal phosphate and 0.012 mM α-ketoglutarate. α-ketoglutarate was omitted from the blank cuvette. The reaction was initiated by the addition of α-ketoglutarate and followed by the rate of increase of absorption of light at 310 mμ in a Cary 14 recording spectrophotometer.

CHAPTER IV

RESULTS

Preparation of Rat Liver Cell Suspensions

A solution of 0.05 % collagenase and 0.10 % hyaluronidase was freshly prepared. The enzymes were dissolved in calcium-free Hanks solution. The rat was treated under light ether anesthesia and the hepatic artery was severed and the liver was back perfused immediately with 8 ml of the cold (5-8^{o}) enzymatic solution using a hypodermic syringe with a no. 25 needle. During the perfusion the pressure was gradually increased until the blanched liver was fully turgid. Then the liver was removed and cut into slices (2 x 2 mm) with scissors. Approximately 2 g of tissue slices were placed in each of two 250 ml beakers or 150 ml flasks containing 6 ml of the enzymatic solution. All procedures to this point were carried out at 4^{o} in an ice-filled container. The beakers were then incubated at 37^{o} in an atmosphere of 95 % O_2 and 5 % CO_2 with constant shaking at 50 oscillations per minute for 60-70 minutes. At least 10 ml of cold calcium- and glucose-free Hanks solution were added to the beakers or flasks and the tissue was disrupted gently by drawing the suspension in and out of a pipette having a tip with an internal bore diameter of 2 mm. The suspension was transferred from beaker or flask to another and transferred back to the original beaker or flask. The resulting suspension was filtered through a single layer of stocking nylon and then through a

stainless steel screen (100 mesh) to remove the clumps. 5-10 ml of additional Hanks solution were used to wash the tissue remaining on the top of the nylon and screen. The cells were sedimented for 1 min. at 50 x g at 4^{o}. The cells were washed twice by resuspending in 10 ml of the Hanks solution followed by recentrifugation. The cells were resuspended in fresh medium for further experiments.

The cells prepared by the above method contained 20-50 % intact cells as revealed by light microscopy with Eosin Y and Trypan Blue as stains. Howard et al. (21) reported that they produced 90-95 % intact cells. However, we were unable to reproduce the results obtained by these authors. The cells showed high respiration (Figure 7).

Induction of Tyrosine Transaminase by Dexamethasone Phosphate

In dispersed rat liver cells, it was observed that tyrosine transaminase was induced by the dexamethasone phosphate, a synthetic glucocorticoid, to a 3-6 fold increase in enzymatic activity. This indicated that some of the cells, if not all, retained the normal function of the whole organ and tissue. The enzymatic level rose slowly after 3.5 to 5.0 hr. (Figures 8 and 9), and then declined. The induction response in the dispersed rat liver cells by the addition of dexamethasone was very encouraging because this type of system will provide a convenient way for studying the nature of the hormonal induction phenomena in normal mammalian cells.

It was also observed that actinomycin D and cylcoheximide partially inhibited the synthesis of the induced tyrosine transaminase when actinomycin D and cycloheximide were added at 1.5 hr. after hormonal treatment. It seemed that cycloheximide inhibited the synthesis of tyrosine

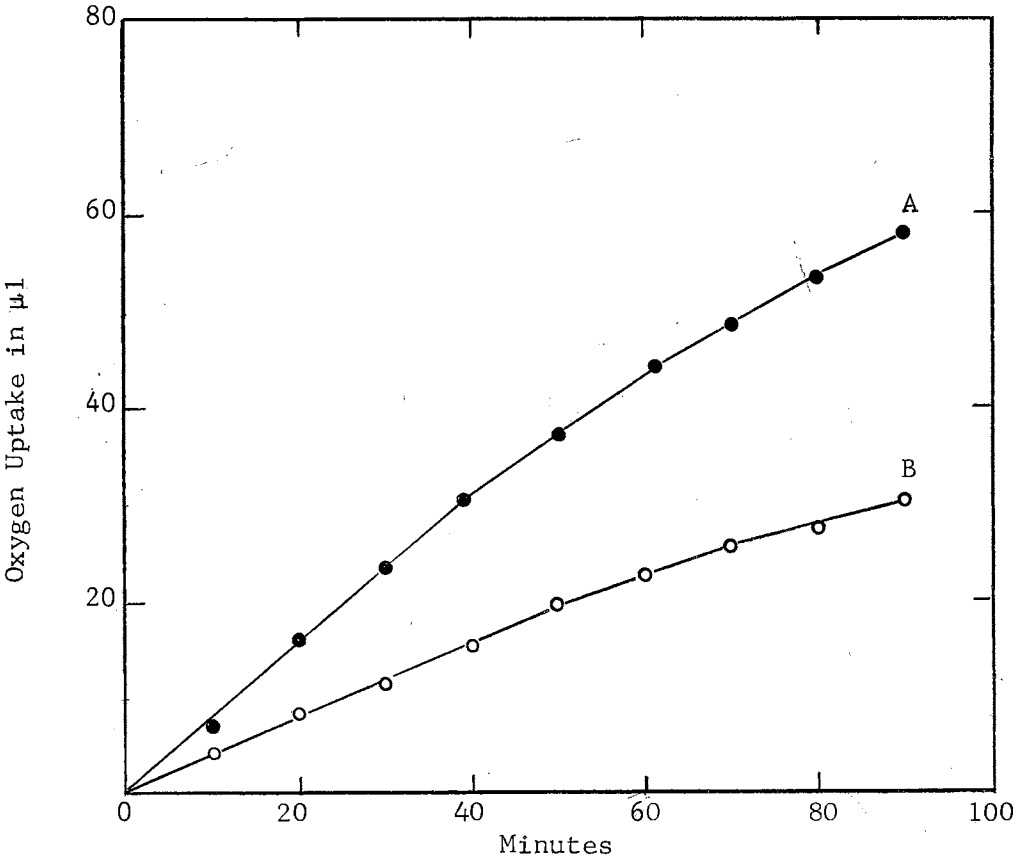

Figure 7. Oxygen Uptake in the Rat Liver Cell Suspension

Respiratory activity of liver cell suspensions in the calcium-free and glucose-free Hanks solution. The concentration of cell suspension in A flask, ●-●, was two fold that of B flask, O-O. The cell suspensions were from the same preparation. 2 ml of cell suspension were used in each of the flasks.

transaminase more readily than actinomycin D (Figure 9). Table I is a summary of the induction and inhibition experiments.

TABLE II

SUMMARY OF INDUCTION AND INHIBITION EXPERIMENTS

Experiment Number	TAT Initial Rate	Rate at Maximum Induction		Induction Fold	Rate at Maximum Induction With Inhibitors		Time at Maximum Rate
	Total units/10 min	No Dex	With Dex		Cycloheximide	Actinomycin D	(Hr)
1	1.2	1.4	3.8 (100%)	2.7			5.0
2	1.53	2.28	5.42	2.37			4.5
3	0.98	1.2	2.86	2.38			4.5
4	0.30	1.2	8.3	6.9	7.0 (84%)	10.2 (122%)	5.0
5	1.9	2.8	13.6	4.95	4.9 (36.1%)	6.2 (45.5%)	4.5
6	0.6	0.6	4.5	7.5	0.95 (23.7%)	2.0 (44.5%)	3.5
7	4.2	2.0	8.8	4.4	4.3 (49.0%)	6.5 (74.0%)	4.0
8	2.4	3.0	11.0	3.66	3.5 (31.7%)	5.0 (45.5%)	7.0

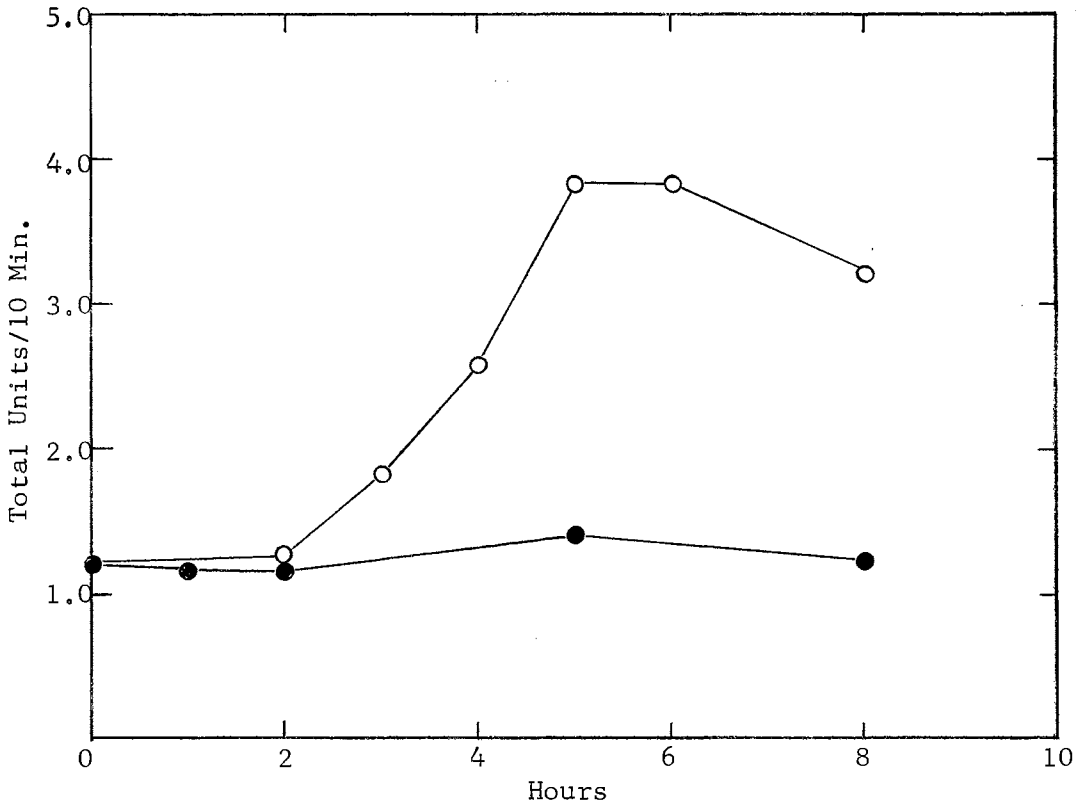

Figure 8. Induction of Tyrosine Transaminase by Dexamethasone Phosphate in the Isolated Rat Liver Cells

O-O, 10^{-5} M dexamethasone phosphate (Dex) was added at zero time and, ●-●, enzyme level without addition of Dex was measured as the control. The dispersed rat liver cells were suspended in calcium-free Hanks solution.

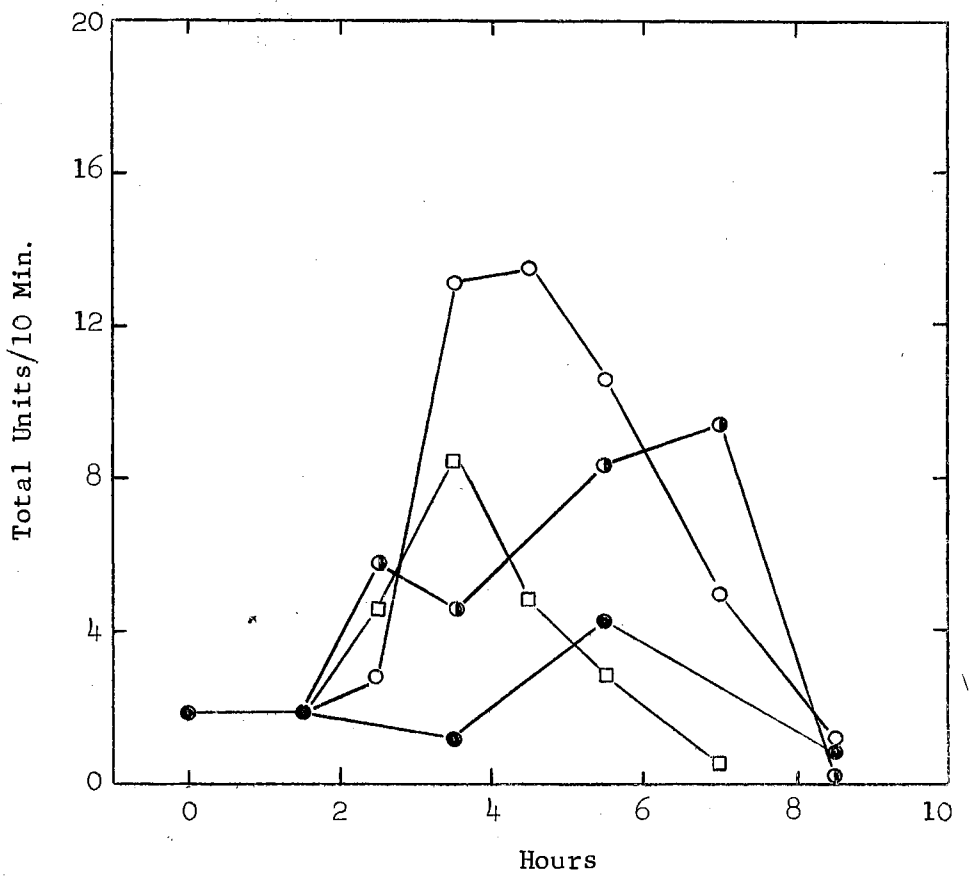

Figure 9. Effect of Dexamethasone Phosphate on the Tyrosine Transaminase and the Effect of Cycloheximide and Actinomycin D on the Induced Tyrosine Transaminase in Rat Liver Cells

O-O, 10^{-5} M dexamethasone phosphate (Dex) was added at zero time. ●-●, control experiment with no hormone added. ◑-◑, 10^{-5} M Dex was added at zero time and 0.50 µg actinomycin D/ml was added after 1.5 hour incubation at 37° C. □-□, 10^{-5} M Dex was also added at zero time and 0.2 µmole/ml cycloheximide was added after 1.5 hour incubation.

CHAPTER V

DISCUSSION

There have been a number of reports on procedures suitable for making cell suspensions using enzymatic digestion (11,12), filtration through a sieve (9), acid treatment in phosphate (10), and perfusion of the tissue with chelating agents. However, these procedures have two major disadvantages for use in biochemical studies. One is the lack of endogenous respiration of the resulting cells, and the other is the loss of soluble enzymes from the cells.

The procedure (20,21) for obtaining suspensions of isolated rat liver parenchymal cells described in this paper gives a preparation containing a high percentage of viable and structurally intact cells as observed under light microscopy. Most of the cells seemed compact and round in the shape. Cells which had irregular and flat shapes were assumed to have damaged membranes as reported by the other investigators (15,20,44).

Large amounts of the dispersing enzymes tended to damage the cells during the course of digestion of the tissue. As a consequence, the amount of enzymes added was reduced to 0.05-0.07 % of collagenase and 0.10 % of hyaluronidase. The cells seemed more compact and round. These results were similar to those found recently by Howard and Pesch (21). They reported that a decreased concentration of the enzymes resulted in a decreased amount of blebbing of the plasma membrane. Although Howard

and Pesch (21) stated that their procedure gave pure preparations of cells, we found that there were considerable small pieces of tissue in the cell suspension.

It appears that the higher respiration rate of the unwashed cells is due to one or more factors which may be lost from the suspension by washing (72). These may include enzymic cofactors, substrates, or stimulating substances released from disrupted cells. The same principle has been adopted for the isolation of intact cells from other tissues such as kidney and tumors. Suspensions of cells obtained as described in this investigation had many advantages over other types of preparations for studying biological reactions at the cellular level.

In 1961, Karlson (73,74) postulated a new mechanism of hormone action. According to this hypothesis, hormones activate certain genes, thus giving rise to specific mRNA and induced protein (enzyme) synthesis. This pheonomenon, gene activation, also known as puffing, can be induced by the insect hormone ecdysone (75). In mammals, evidence for gene activation can only be indirect. In studying the mechanism of action of cortisol, Lang et al. (76) directed their attention to the stimulation of mRNA synthesis. By the use of the phenol extraction method of Georgiev and Mantieva (77), and a very sensitive enzyme assay using radioactive substrate, they were able to postulate that a mRNA fraction contains the information for the formation of tyrosine transaminase and that the enzyme is formed in vitro by de novo synthesis. Development of enzymatic activity is dependent on active protein synthesis and is inhibited by puromycin and erythromycin.

Tyrosine transaminase was induced by the dexamethasone phosphate in dispersed liver cells isolated under the procedures (20,21) described.

This suggested that the cells as isolated do exhibit normal liver function. Tyrosine transaminase is a soluble enzyme and the fact that it could be induced in isolated liver cells would indicate that the cells had normal membranes.

Previous reports from this laboratory (2) indicated that the failure of the induction in the isolated cells was probably due to the loss of soluble components from the cells. This is the first example of the induction of an enzyme in a normal isolated cell.

The activity of tyrosine transaminase was variable. This may have been partly due to variability in the percentage of viable cells in the different preparations.

CHAPTER VI

SUMMARY

The cell suspensions were prepared by the procedure of Howard *et al.* (20). The cell preparations contained approximately 20-50 % viable cells as revealed by light microscopy. Induction of tyrosine transaminase was observed in rat liver cells by the dexamethasone phosphate (Dex), a synthetic glucocorticoid. Dex increased enzymatic activity 3-6 fold. The success of the induction indicated that dispersed rat liver cells maintained the normal function and properties of cells *in situ*. Actinomycin D and cycloheximide exhibited partial inhibition on the induction of tyrosine transaminase by dexamethasone phosphate.

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