I. CORTISOL BINDING AND RETENTION OF LACTATE

DEHYDROGENASE IN RAT LIVER CELL SUSPENSIONS

II. STABILIZATION, PURIFICATION AND

PROPERTIES OF BOVINE MAMMARY

UDP-GALACTOSE 4-EPIMERASE

By

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PART I

PART II

GENERAL INTRODUCTION

The first part of this dissertation is concerned with the assess-
ment of rat liver cells in suspension as a potential system for study-
ing the in vitro induction of tyrosine transaminase (EC 2.6.1.5) by
glucocorticoids. It was found that the isolated liver cells did not
respond to glucocorticoids and the reason for this lack of response
was probably due to the fact that the prepared cells had damaged
membranes and as a result the soluble cellular components diffused
into the suspending medium. It was clear that a different method for
preparing the cells was necessary so that the cellular membranes were
not damaged. Attempts were made to prepare cells in different ways
but were unsuccessful and as a result the problem was terminated at
this stage.

The second part of the dissertation is concerned with the
stabilization, purification and properties of bovine mammary UDP-
galactose 4-epimerase (EC 5.1.3.2), an enzyme associated with the
biosynthesis of lactose. This enzyme was purified to near homogeneity
and its properties were documented.

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PART I

CORTISOL BINDING AND RETENTION OF LACTATE DEHYDROGENASE

IN RAT LIVER CELL SUSPENSIONS

PART I

CHAPTER I

INTRODUCTION

Liver cells have been isolated by various methods, such as forcing
the tissue through stainless steel sieves (1), tryptic digestion (2)
and incubation in acidic solution (3). Since Anderson (4) obtained
a high yield of isolated cells, there has been considerable interest
in the metabolic properties of dispersed rat liver cells and attempts
have been made to demonstrate normal liver function in these cells
since they would provide an attractive system for the study of liver
function. However, satisfactory preparations of intact, dispersed
cells have been difficult to achieve. The cell suspensions produced
were partly damaged during the isolation procedures which resulted in
low rates of endogenous respiration and enzymatic leakage. Tests of
enzymatic activities showed a large loss of various enzymes, especially
those of the soluble fraction of the cell, to the suspending medium.
Enzymatic leakage did not occur during the perfusion of the liver but
resulted from the dispersal process. The leakage of some enzymes
may be partly overcome by pretreatment of the animal with glucocorti-
coid hormones (5), but this method is not entirely satisfactory.
Therefore, before the biological activities in intact cells can be
studied, it will be necessary to overcome these obstacles and obtain

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a cell dispersion which allows for a more normal metabolic activity.
The potential use of isolated liver cells to study normal liver func-
tions, especially the hormonal induction of enzymes, prompted the
present studies. In addition, the binding of cortisol and the reten-
tion of soluble enzymes in the isolated cells were examined. Lactate
dehydrogenase may be retained in isolated rat liver cells under certain
conditions, whereas other soluble enzymes are lost during the prepara-
tion procedures. The isolated liver cells incorporate 4 - 5% cortisol
from the medium and about 75% of the cortisol in the cell is associated
with the particulate fraction.

CHAPTER II

LITERATURE REVIEW

Preparation of Isolated Liver Cells

Liver cell suspensions were first prepared by Schneider and Potter (6) in 1943 by forcing a liver through cheese cloth. Centrifugation at low speed resulted in separating the whole cells from the cellular debris and red blood cells. Kaltenbach (1) passed rat liver through stainless steel gauzes of increasingly fine mesh. Zaroff et al. (7) reported a method in which rat liver was chopped in an automatic slicer in two directions at right angles to each other, at a setting designed to give 50 μ thick slices. The clumps thus obtained were removed by repeated passage through a narrow needle. Liver cells were obtained in suspension by LePage (8) by passing minced tissue through a tissue press. Bucher (9) obtained rat liver cells by shaking liver fragments with glass beads and the average yield of free cells was about 10%.

Anderson (4) carried out preliminary perfusion of the liver in order to reduce the adhesiveness of the cells. Rat liver was perfused with calcium binding agents, like citrate, pyrophosphate, versene (EDTA)[*] or ATP, in calcium-free Lock! solution, and the perfused liver

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[*]All abbreviations are in accordance with the IUPAC-IUB combined Commission on Biochemical Nomenclature. J. Biol. Chem., 241, 527 (1966).

was homogenized gently in a homogenizer with a loose fitting Leucite pestle. The crude cell suspension was passed through cheese cloth to remove clumps and shreds of connective tissue, and then centrifuged several times to remove broken cells. Anderson's method or with minor modification has been used by many authors (10, 11, 12). Rutter and Brosemer (13) used a method in which rat liver was perfused with a solution containing sucrose and citrate, or with a nutrient medium containing bovine serum albumin but no citrate or carbohydrate. The perfused liver was suspended in the medium of Dulbecco and Vogt (2) and chopped with a battery of razor blades. The resulting cell suspension was passed through sieves of increasing mesh and then centrifuged several times to remove contaminants.

In the method of Branster and Morton (14) rat liver may be perfused with a variety of perfusing agents containing no chelating agent, like 0.9% sodium chloride, Lock solution (with or without calcium), isotonic or slightly hypertonic sucrose solution, polyvinylpyrrolidone or serum. The ease of isolation of the cells in suspension increased with increasing temperature of perfusion up to 50°C. The optimum temperature recommended was 37°C. The dispersion of cells was achieved in a homogenizer with a polyethylene pestle. The preparation was passed through bolting nylon of increasing mesh to remove large clumps and strands of connective tissue. The broken cells were removed by centrifugation. Branster and Morton's method (14) was used by Berry (15) and Exton (16). A similar method was used by McGeachin and Potter (17) in which rat liver was perfused, minced and passed through a Leucite press and then filtered several times through screens of 30,

50 and 60 mesh, the broken cells were removed by six centrifugations
at 1,500 r.p.m., each for 10 minutes, followed by a final centrifuga-
tion at 3,000 r.p.m. for 10 minutes.

Longmuir and Rees (3) incubated chopped rat liver at 37°C in an
acidic medium (pH 5), and the tissue was disrupted by drawing it 50
times through an inverted pipette. The clumps were removed by filtra-
tion, and the blood cells and cellular debris were removed by several
centrifugations.

In 1966, Rappaport and Howze (18) reported on the dissociation of
mouse liver _in vitro_ into a suspension of single cells by using sodium
tetraphenylboron. Sodium tetraphenylboron is an agent which specially
binds K^+ ion and does not bind the divalent cations. They suggested
that K^+ ion might be the major cation promoting cell-cell adherence in
liver.

The enzymatic digestion of intercellular material has been used
in the preparation of cell suspensions. Tryptic digestion of minced
small fragments of kidney was used in the preparation of kidney cell
suspensions (2). Collagenase was employed in the preparation of cells
from adipose tissue (19). The preparation of epithelial cells from
rat intestine with hyaluronidase digestion was also reported (20).
With combination of collagenase and hyaluronidase, isolated rat liver
cells were recently prepared by Howard and Pesch (21, 22). From the
vital stain and endogenous respiratory rate, the isolated rat liver
cells prepared by collagenase and hyaluronidase digestion are far
superior to the cells prepared by other methods.

The above methods can be classified as follows: (a) methods with
mechanical treatment of the tissue, (b) methods in which the liver is

perfused with a chelating agents such as EDTA or sodium tetraphenyl-
boron to remove calcium or potassium from the intercellular cement,
followed by a mild mechanical treatment, (c) methods involving a
chemical or physical treatment to soften or to remove the intercellular
cement, (d) an enzymatic treatment of the liver to degrade a polymeric
component of the intercellular cements.

Morphology of Isolated Cells

Rat liver cells obtained by collagenase and hyaluronidase diges-
tion were round and compact and had a well defined outline under the
light microscope. In contrast, the cells prepared by other methods
were elliptical, larger, and the cell outline was less clear under the
light microscope (21). A few reports on fine structure of cells have
been published. In all the preparation methods except Howard and Pesch
(21), the cells had an altered fine structure. The endoplasmic
reticulum was in disarray and had round up to vesicles. The
mitochondrial matrix was swollen or contracted and the nuclei were
badly swollen (23). In most cases, internal damage was observed, and
the plasma membrane was not intact (23). In the Howard and Pesch
preparation (21), the nucleus, mitochondria and endoplasmic reticulum
all appeared essentially normal as compared to cells in whole liver.

Function of Isolated Liver Cells

Branster and Morton (14) reported a low endogenous respiration in
their cell suspension. Kalant and Young (24) reported that isolated
liver cells had remarkably low endogenous respiration and that
succinate was the only substrate which could be metabolized. Later,

Exton (16, 25) showed that rat liver suspension did not metabolize carbohydrates but did metabolize citric acid cycle intermediates and oxidize palmitate largely to ketone bodies. Ichihara et al. (26) found that respiration was remarkably stimulated by the addition of ADP, and also showed that ^{14}C-acetate was incorporated into lipids of dispersed rat liver cells. Berry (15) had prepared mouse liver cell suspensions and showed that these cells were able to synthesize urea from ammonium chloride. Jacob and Bharagava (27) examined the incorporation of ^{32}P-phosphate and ^{14}C-adenine into RNA of suspended liver cells.

A serious limitation of isolated cell preparations has been their failure to utilize glycolytic intermediates (16), and many of the soluble components of the cells, including enzymes, are lost during the preparation procedure. Scuh losses have been attributed to alteration in the permeability of the cell membranes as evidenced by election microscopy (23). The soluble enzymes, aldolase, lactate dehydrogenase, iditol dehydrogenase (16), threonine dehydrase, serine dehydrase, tryptophan pyrrolase (5) and phosphorylase (28) were lost from rat liver cells during the preparation of cell suspensions. Glutamic pyruvate transaminase (5), malate dehydrogenase (16), glutamic oxaloacetic transaminase, glutamate dehydrogenase and glucose-6 phosphatase (29), are enzymes associated with the particulate portion of the cells and were retained to varying degrees in dispersed liver cells. Nicotinamide nucleotides are essentially lost during the preparation of liver cells. Takeda et al. (5) has shown that the leakage of glutamic pyruvic transaminase and lactic dehydrogenase

could be prevented by pretreatment of the animals with glucocorticoid hormones. In Howard and Pesch's (23) cell suspensions, the endogenous rates of respiration were significantly higher than other methods, and nearly linear respiration rates were observed for 2 hours. Respiration was also greatly stimulated by 1% albumin (22).

Cortisol Binding in Cells and Tissues

Cortisone or cortisol rapidly leaves the blood of man and animal following intravenous administration. Migeon et al. (30) found that liver contained much higher cortisol-4-^{14}C than other tissues. The liver removes corticosteroids from the blood stream and cleaves them enzymatically (32). Levin et al. (32) reported that cortisol was bound and degraded rapidly by rat liver slices. Diaphram, kidney and spleen also bound cortisol but had substantially less ability to degrade the steroid. Ketchel (33) presented evidence that human leucocytes were able to bind cortisol in vitro. Eighty percent or more of the total cortisol was taken up within 5 minutes and equilibrium was established within 10 to 15 minutes. The cortisol bound by leucocytes had the physiological effect of inhibiting the amoeboid migration of the leucocytes. Bellamy et al. (31) reported that in rat liver slices cortisol entered the cell by simple diffusion and a gradient was maintained by intracellular adsorption. Cortisol was slowly concentrated by liver slices and after a period of 6 hours the cortisol concentrated in the slices was about six times higher than that in the incubation medium.

Jacob and Bharagava (27) demonstrated the incorporation of

^{32}P-phosphate and ^{14}C-adenine into RNA of suspended rat liver cells. However, the initial lag in the incorporation of adenine into RNA or the cell suspensions was much longer than tissue slices. Lin and Knox (34) reported that glucocorticoids increased the activity of tyrosine transaminase in rat liver. Kenney (35) presented the evidence that induction of tyrosine transaminase was involved in the increase rate of enzyme activity. Tomkins et al. (36, 37) demonstrated that a glucocorticoid stimulated at 5 to 15 fold increase in the activity of tyrosine transaminase in hepatoma tissue culture cells. Immunochemical and inhibitor experiments indicated that the glucocorticoids caused an increase in the number of enzyme protein molecules and induced an increase in the level of tyrosine transaminase m-RNA.

CHAPTER III

MATERIALS AND METHODS

Materials

Holtzman rats were obtained from Holtzman Co., Madison, Wisconsin. Cortisol-4-^{14}C, specific activity 25 mc/mM, was purchased from New England Nuclear Corp. The purity was checked by ascending co-chromatography with non-radioactive cortisol on Whatman No. 1 developed by the solvent system, benzene : methanol : water (2:1:1). The radio-activity matched with the only spot detected by an ultra violet lamp (254 mμ). Cortisol-4-^{14}C, 72.5 μg and specific activity 25 mc/mM, was dissolved in 1 ml ethanol and was used in the experiments. In certain experiments the radioactive cortisol was diluted with cortisol to give a final concentration of 0.5 μg/ml.

Succinate disodium salts, tyrosine, α-ketoglutarate and trihydroxylmethylaminomethane (Tris) were obtained from Sigma Chemical Co.; ethylenediamino tetraacetic acid disodium salt (EDTA) and methylcellulose were from Matheson Coleman and Bell Co.; polyvinyl-pyrrolidone (PVP) from General Aniline and Film Corp.; 3-hydroxy-anthranilic acid was a gift from Dr. R. K. Gholson, Oklahoma State University. All other chemicals used were of reagent grade. The following solutions were used for the perfusion, dispersion and incubation of rat liver cells:

1. Perfusion solution: sucrose 0.25 $\underline{M}$, 1 m$\underline{M}$ EDTA, pH 7.3.

2. Dispersion media:

    a). 0.25 $\underline{M}$ sucrose, pH 7.3.

    b). 0.25 $\underline{M}$ sucrose, 0.02 $\underline{M}$ Tris-HCl, pH 7.3.

    c). 0.25 $\underline{M}$ sucrose, 0.02 $\underline{M}$ potassium phosphate, pH 7.3...

    d). 0.25 $\underline{M}$ sucrose, 0.4% methylcellulose, pH 7.3.

    e). 0.25 $\underline{M}$ sucrose, 0.4% methylcellulose, 0.02 $\underline{M}$ Tris-HCl, pH 7.3.

    f). 0.22% polyvinylpyrrolidone (w/v), 10% sucrose (w/v), pH 7.3.

3. Incubation medium: sucrose 0.25 $\underline{M}$, Tris-HCl 20 m$\underline{M}$, KCl 20 m$\underline{M}$ and disodium succinate 10 m$\underline{M}$, pH 7.3.

Methods

Preparation of Rat Liver Cell Suspensions

Liver cell suspensions were prepared from Holtzman rats weighing between 150 and 300 grams. The animals were put into a glass jar containing a cotton pad presoaked with ether. As soon as the rat was anesthetized it was removed out of the jar and laid on its back in a shallow plastic tray. The body cavity was cut open with a pair of sharp scissors and the liver was perfused via the portal vein with 75 ml to 100 ml of the perfusion solution at 37°C. The following perfusion process was used. After the syringe needle was inserted into portal vein, the vessels between liver and heart were cut with the scissors. Blood was forced out of the liver with the perfusion solution maintained at pressure of 50 cm. The liver was removed

and cut with the scissors into small pieces. They were forced gently
through a Harvard tissue press. The minced tissue was dispersed in
various dispersion media at 4°C in a loose fitting teflon pestle on
glass homogenizer (A. Thomas, type B, teflon pestle and a 19.5 mm
internal diameter test tube was used). 9 ml of the dispersion medium
were used for each 2 gm of liver. The dispersion operation should be
as gentle as possible in order to disperse the minced tissue. The
crude cell suspensions were obtained by filtering through two layers
of cheese cloth which removed connective tissue and large clumps of
cells. The filtrate was centrifuged at 120 x g for 5 minutes at 4°C.
The supernatant solution was designated as cell supernatant. The cell
pellet was washed twice with two volumes of the dispersion medium and
the cells were resuspended in 5 ml of the incubation medium. All
procedures except the perfusion were performed in an ice-bath.

Measurement of Oxygen Consumption

Oxygen consumption was measured in a conventional Warburg
apparatus (38). Each flask contained 3 ml of incubation medium in
the main compartment, 0.5 ml of the cell suspension in the side arm,
an 0.2 ml of 10% (w/v) KOH in the center well. After the flasks were
equilibrated at 37°C for 10 minutes, the cell suspension in the side
arms was tipped into the main compartment, and the oxygen uptake was
recorded every 15 minutes for 4 hours.

Binding of Cortisol-4-^{14}C

Cortisol-4-^{14}C Uptake by Cell Suspensions

Cortisol-4-^{14}C, dissolved in a minimum volume of ethanol, was added to the cell suspensions which were incubated at 37°C for 20 minutes. After the incubation period the cells were centrifuged in a clinical centrifuge tube at 300 x g for 5 minutes and washed once with the dispersion media. The cell pellet was solubilized in 1 ml of 70% nitric acid at 70°C (39). Aliquots were put on aluminium planchets (diameter 3.1 cm) and dried. Radioactivity was counted at infinite thickness in a Baird Atomic Automatic Planchet Counter.

Distribution of Radioactivity in the Cells

After the cell suspensions were incubated with cortisol-4-^{14}C for 20 minutes, the cells were washed and resuspended in 0.25 $\underline{M}$ sucrose. The radioactive cell suspensions were homogenized and subjected to subcellular fractionation. The nuclear fraction was centrifuged down at 700 x g for 10 minutes. the mitochondrial fraction at 5,000 x g for 10 minutes, the microsomal fraction at 57,000 x g for one hour, the soluble fraction remained in the supernatant solution (40). Aliquots of various fraction dissolved in 70% nitric acid were dried on planchets and the radioactivies were counted with the Baird Atomic Planchet Counter at infinite thickness.

Enzymatic Assays

Enzymatic activities were measured by spectrophotometric methods.

Absorbance changes were recorded by a Cary model 14 spectrophotometer. All assays were proportional to the amount of enzyme in the extracts.

Lactate Dehydrogenase (EC 1.1.1.27)

The assay used is described in Methods in Enzymology (41). The oxidation of NADH is a direct measurement of the reduction of pyruvate to lactate which can be observed at 340 mμ. The reaction mixture contained 1 μmole of pyruvate, 0.2 μmoles NADH, 100 μmoles phosphate buffer at pH 7.4, and a suitable amount of enzyme and water to make the final volume of 3.0 ml.

Tyrosine α-ketoglutarate Transaminase (EC 2.6.1.5)

The method of Lin and Knox was used (42).

$$\text{L-tyrosine} + \alpha\text{-ketoglutarate} \Longleftrightarrow \text{p-hydroxyphenylpyruvate} + \text{L-glutamate}$$
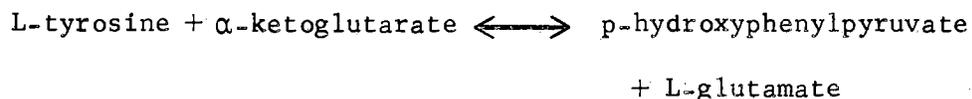
The reaction is followed by determining the formation of p-hydroxyphenylpyruvate. Tautomerase (EC 5.3.2.1) catalyzes the enol-keto tautomerization of p-hydroxyphenylpyruvate. The enol is stabilized in borate buffer by the formation of a complex which absorbs intensively at 330 mμ. In the presence of excess tautomerase, the activity of the tyrosine transaminase is measured under first order conditions. The p-hydroxyphenylpyruvate tautomerase was prepared from hog kidney by the method of Knox and Pitt (43).

3-hydroxyanthranilate Oxygenase (EC 1.99.2.b)

The formation of compound I from 3-hydroxyanthranilate results

in an increase of absorbance at 360 mμ. The reaction mixture contained 280 μmoles of Tris, pH 7.0, water and enzyme. The reaction was started by the rapid addition of 0.1 ml of $3 \times 10^{-3}\underline{M}$ 3-hydroxyanthranilic acid solution. The final volume was 3.0 ml and the enzymatic activity was measured by following the increase of absorbance at 360 mμ (44).

CHAPTER IV

RESULTS

Respiratory Activity of Rat Liver Cell Suspensions

An aliquot of 0.5 ml cell suspension was centrifuged at 150 x g

for 5 minutes and the supernatant solution was removed with a pipette.

The residue of cells was resuspended in 3 ml of incubation media con-

taining 0.01 $\underline{M}$ sodium succinate as substrate in 0.25 $\underline{M}$ sucrose and

transfered to the Warburg respirometer. The endogenous respiration

as shown in Figure 1, curve A was very low in the control flask with-

out substrate. In the presence of sodium succinate and 0.02 $\underline{M}$ KCl

the oxygen uptake was linear for about 60 minutes as shown by curve B,

and with 0.08 $\underline{M}$ KCl as shown by curve C. The oxygen uptake was the

same as the control (no glucose) when glucose was used as a substrate.

The Time Course of Cortisol Uptake
by Rat Liver Cell Suspensions

After 10 μl cortisol-4-^{14}C was added to 7 ml of a rat liver cell

suspension preincubated at 37°C, aliquots of 1 ml cell suspension were

taken out at various periods of time. The aliquots of the cell suspen-

sion were centrifuged, washed and dissolved in 70% nitric acid and the

radioactivity was counted. The results are shown in Figure 2.

The uptake of cortisol-4-^{14}C by rat liver cell suspension reached

its maximum in 20 minutes though most of the total cortisol was taken
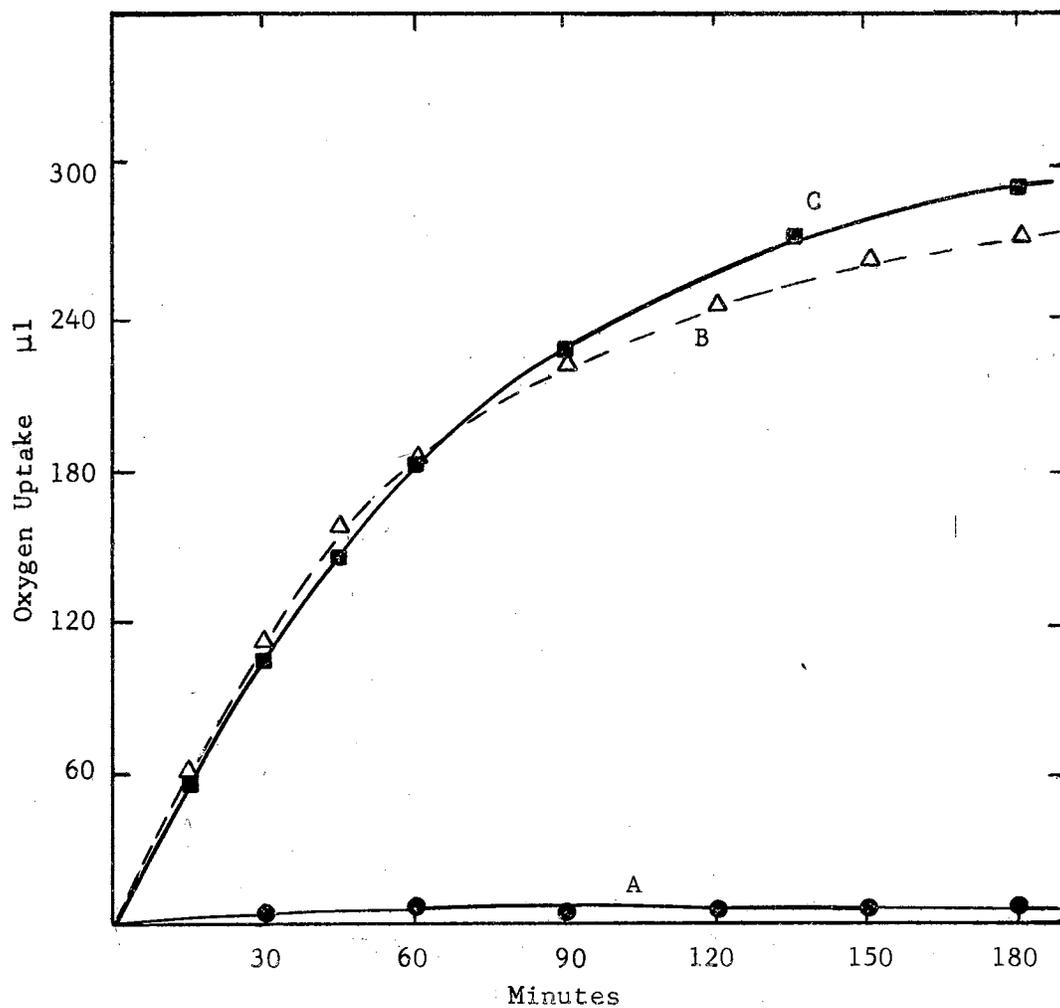
Figure 1. Oxygen Uptake of Rat Liver Cell Suspension.

The oxygen uptake of cells originating from 0.5 ml of cell suspension in media containing 0.25 M sucrose, 20 mM Tris, pH 7.3, 10 mM succinate, 20 mM KCl (curve B, △—△), or 80 mM KCl (curve C, ■—■), and in the control medium without succinate (curve A, ●—●).
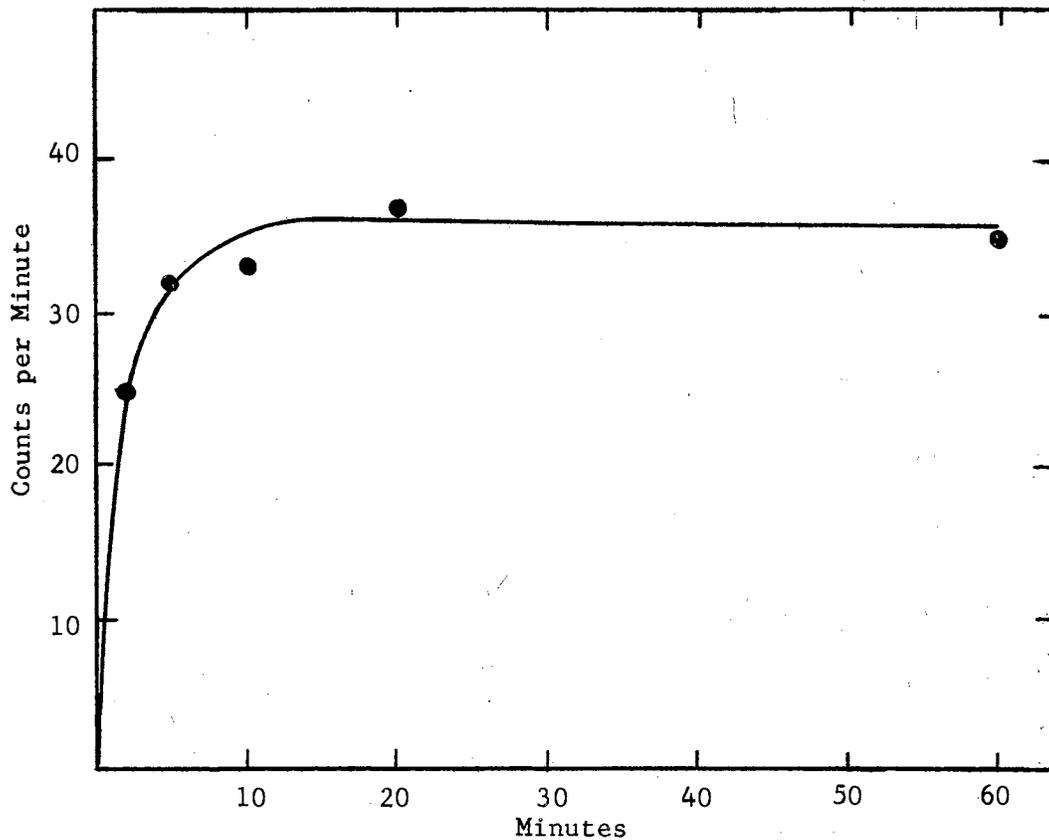
Figure 2.   Time Course of Cortisol Uptake by Rat Liver Cell
            Suspension.

10 µl of cortisol-4-^{14}C were added to 7 ml of a rat
liver cell suspension preincubated at 37°C. Aliquots of 1 ml
cell suspension were taken out after 2, 5, 10, 20 and 60
minutes. The cell suspension was centrifuged, washed and
dissolved in 70% nitric acid and the radioactivity was counted.

up within the first 5 minutes. The results are similar to studies on the cortisol uptake by Leucocytes (30).

The Effect of Concentration on Cortisol Uptake

Various amounts of cortisol-4-^{14}C were added to each ml of cell suspension. The amount of cortisol uptake was indicated by the amount of radioactivity incorporated into the cells. After incubation at 37°C for 20 minutes, the cell suspensions were centrifuged, washed once, dissolved in 70% nitric acid and the radioactivity was counted. There was a linear relationship between cortisol uptake and concentration in the range of 1 - 10 μg of cortisol per ml of cell suspension as shown in Figure 3.

The Effect of Cell Concentration on Cortisol Uptake

The relationship between cortisol uptake and cell concentration is shown in Figure 4. There was a linear relationship between the uptake of cortisol and the amount of cells in the medium over an eight fold range of cell concentration when the cortisol concentration was 0.7 μg per ml.

Incorporation of Cortisol into Rat Liver Cell Suspensions

Cortisol-4-^{14}C, 0.7 μg and specific activity 25 mc/mM, was added to each 1 ml of cell suspension and incubated at 37°C for 20 minutes. The cell suspensions were centrifuged and washed once with 2 ml of dispersion medium. The supernatant portion, wash portion, and the cell residues were dissolved in 70% nitric acid. The radioactivities
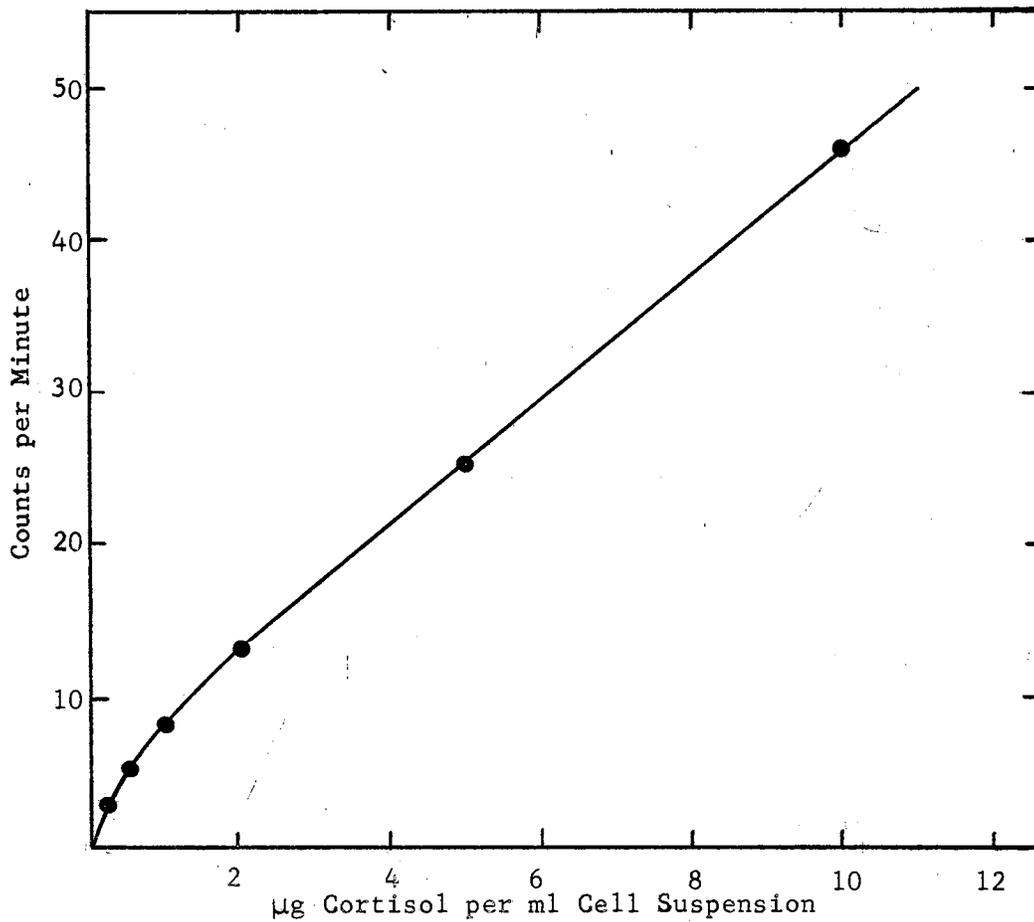
Figure 3. Effect of Concentration on Cortisol Uptake.

Various amounts of cortisol-4-^{14}C, 0.5 µg/µl and specific activity 3.6 mc/mM, were added to each ml of cell suspension. Cortisol uptake was indicated by the amount of radioactivity incorporated into the cells. After incubation at 37°C for 20 minutes the cell suspensions were centrifuged, washed once, dissolved in 70% nitric acid and the radioactivity was counted.
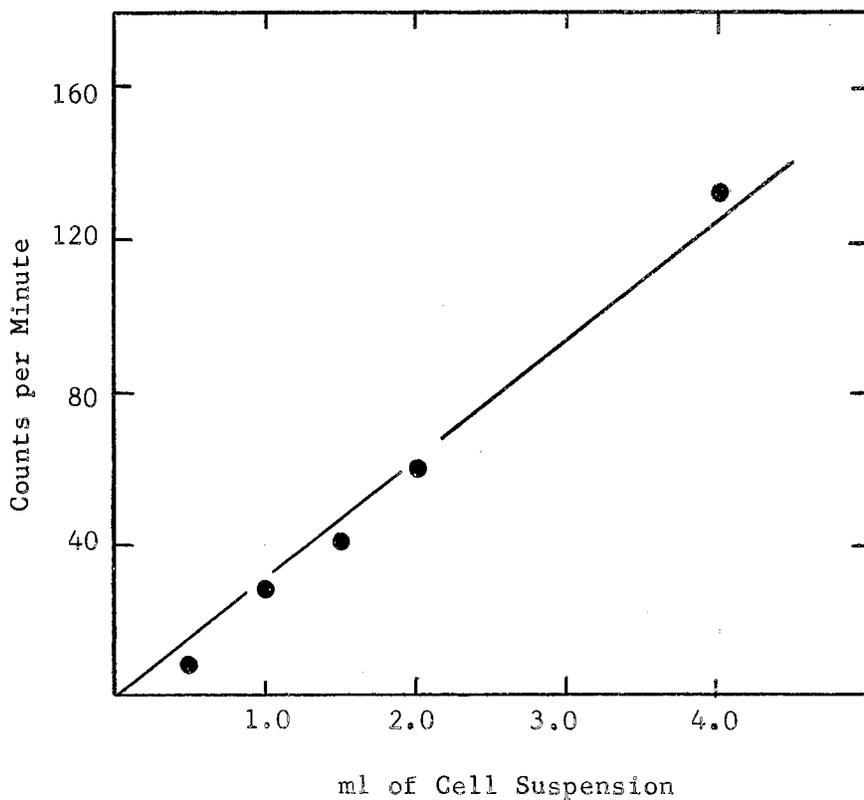
Figure 4. Effect of Cell Concentration on
Cortisol Uptake.

0.5, 1, 2, and 4 ml of cell suspension were
placed in five conical centrifuge tubes, centri-
fuged and resuspended in 1 ml of dispersion medium.
10 µl of cortisol-4-^{14}C, 0.7 µg and specific acti-
vity 25 mc/mM, were added to each tube. After in-
cubation at 37°C for 20 minutes, the tubes were
centrifuged, the cell residues were washed and dis-
solved in 70% nitric acid and the radioactivity was
counted.

of these three portions were counted and the results are presented in Table I. The results show that the incorporation of cortisol from the medium into rat liver cell suspensions was about 4 -5%.

Subcellular Distribution of Cortisol
in Suspended Rat Liver Cells

Rat liver cell suspensions were incubated with cortisol-4-^{14}C at 37°C for 20 minutes. They were washed twice with the dispersion medium and resuspended in 0.25 $\underline{M}$ sucrose. The cells were homogenized in a Potter-Elvenhjem homogenizer and the subcellular fractions were isolated by differential centrifugation. The scheme for the subcellular fractionation is shown in Figure 5.

The average distribution of cortisol-4-^{14}C in four experiments as shown in Table II was: nuclear, 36.4% (30.8 - 42.6); mitochondrial, 20.6% (14.2 - 27.7); microsomal, 16.5% (14.4 - 17.9); and soluble, 26.5% (18.8 - 39.0). The values shown in the brackets are the observed ranges.

The Retention of Lactate Dehydrogenase
in Dispersed Rat Liver Cells

Previous studies in this laboratory (45) showed that tyrosine transaminase was lost during the preparation of liver cells . Such losses of soluble cellular components have been attributed to alteration of the normal membrane permeability of isolated cells. Since the cells lost most of their tyrosine transaminase and presumably other soluble components, attempts were made to find a new preparation method that would lead to the preparation of intact cells with

TABLE I

INCORPORATION OF CORTISOL-4-^{14}C INTO RAT
LIVER CELL SUSPENSIONS

Experiment	% Radioactivity		
	Supernatant	Wash	Cells
1	81.0	15.1	4.1
2	82.0	13.1	4.7
3	80.2	15.9	4.3
4	82.5	12.5	4.5

10 ml cell suspension
+ 50 µl cortisol-4-^{14}C (3.5 µg, specific
                          activity 25 mc/mM)

Wash twice with dispersion medium

Resuspended in 10 ml 0.25 M sucrose

Homogenization with Potter-Elvenhjem Homogenizer

Homogenate
700 x g, 10 min.

Residue

Resuspended in 4 ml 0.25 M sucrose
700 x g, 10 min.

Nuclear fraction   Supernatant

Dissolved in 2 ml
nitric acid

Supernatant
5,000 x g, 10 min.

Residue   Supernatant
                                 57,000 x g, 1 hr.

Resuspended in 3 ml 0.25 M sucrose   Microsomal   Supernatant
24,000 x g, 10 min.   fraction   fraction

Mitochondrial   Supernatant
fraction   Dissolved in   Dissolved in
                                      1 ml nitric   1 ml nitric
Dissolved in 1 ml   acid   acid
nitric acid

Figure 5. Scheme for the Subcellular Fraction of
          Rat Liver Cell Suspensions.

TABLE II

DISTRIBUTION OF CORTISOL-4-^{14}C IN SUBCELLULAR FRACTIONS

Experiment	Fraction			
	Nuclear	Mitochondrial	Microsomal	Supernatant
	%	%	%	%
1	42.6	19.0	14.3	24.0
2	30.8	14.2	16.0	39.0
3	36.6	21.8	17.6	24.0
4	35.6	27.7	17.9	18.8
Average	36.4	20.6	16.5	26.5

improved membrane permeability. Lactate dehydrogenase was chosen as an enzyme to detect whether the isolated cells had improved permeability since this enzyme has high activity in liver, the assay is rapid and simple and 95% of lactate dehydrogenase was reported to be lost during the preparation of isolated cells (5).

Liver cell suspensions were prepared with various dispersion media. The crude liver cell suspensions were centrifuged and the supernatant solution was designated as the supernatant portion. The cell pellet was washed twice with the dispersion media. The washing solutions were designated as Wash-I and Wash-II. The final cell pellet was resuspended in the dispersion medium and was designated as the cell portion. The distribution of lactate dehydrogenase activity in these three portions prepared in 0.25 M sucrose is summarized in Table III.

The effect of various dispersion media on the retention of lactate dehydrogenase in dispersed liver cells is shown in Table IV. The data presented in Table IV indicate that 40 to 57% of lactate dehydrogenase was retained in the cells when 0.25 M sucrose alone or in conjunction with 0.4% methylcellulose or 22% polyvinylpyrrolidone was present in the dispersion media. However, the presence of 20 mM Tris or phosphate at pH 7.3 in the media resulted in cells that retained only 1 to 10% of their lactate dehydrogenase.

Retention of Other Soluble Enzymes in Dispersed Rat Liver Cells

The retention of other soluble enzymes was examined in order to determine whether the preparation procedure did produce cells with more intact cell membranes or whether the results were unique to

TABLE III

DISTRIBUTION OF LACTATE DEHYDROGENASE IN THE SUPERNATANT,
TWO WASHES, AND THE CELL PELLET FROM THE CRUDE CELL
SUSPENSIONS IN 0.25 $\underline{M}$ SUCROSE pH 7.3

Rat	Cell Suspensions	Supernatant	Wash-I	Wash-II	Washed Cells
	%	%	%	%	%
1	100	72.0	4.0	2.0	22.0
2	100	69.3	2.6	1.1	28.1
3	100	59.6	4.1	2.2	34.2
4	100	32.0	4.6	1.8	61.6
5	100	43.5	5.6	2.2	49.2
6	100	43.0	6.5	3.0	47.5
7	100	53.3	7.5	2.8	36.5
8	100	64.8	4.8	1.5	29.0
9	100	63.3	3.4	1.4	32.0
10	100	43.9	4.9	1.9	49.4
11	100	44.8	2.2	0.8	52.2
12	100	39.1	4.3	1.5	55.1
13	100	67.8	6.2	0.9	25.1
14	100	47.5	5.7	1.3	46.5
15	100	70.5	7.3	1.2	21.1
16	100	37.1	6.3	2.4	54.2
Average	100	53.1	5.0	1.7	40.2

TABLE IV

EFFECT OF DISPERSION MEDIA ON THE RETENTION OF LACTATE
DEHYDROGENASE IN DISPERSED LIVER CELLS

Exp.	Dispersion Media	No. of Exp.	Average % Lactate Dehydrogenase in Cells
1	0.25 M sucrose, pH 7.3	16	40.2
2	0.25 M sucrose, 0.02 M Tris-HCl, pH 7.3	4	7.7
3	0.25 M sucrose, 0.02 M potassium phosphate, pH 7.3	1	1.4
4	0.25 M sucrose, 0.4% methylcellulose, pH 7.3	1	40.5
5	0.25 M sucrose, 0.4% methylcellulose, 0.02 M Tris-HCl, pH 7.3	1	10.1
6	Polyvinylpyrrolidone (22%), Sucrose (10%), pH 7.3	2	57.5

lactate dehydrogenase. Rat liver cell suspensions were prepared in 0.25 M sucrose, pH 7.3, and lactate dehydrogenase, 3-hydroxyanthranilate oxygenase and tyrosine transaminase retention were measured. The results are presented in Table V.

The results in Table V show that lactate dehydrogenase was retained 40.2% whereas only 2.2% of 3-hydroxyanthranilate oxygenase and 3.9% of tyrosine transaminase were retained under identical isolation conditions. This observation suggests that the retention of lactate dehydrogenase was unique and that the isolation procedures did not produce cells with improved permeability characteristics.

<div align="center">

The Retention of Lactate Dehydrogenase in Cells
As a Function of the Ionic Strength
of the Dispersing Media

</div>

The data in Table IV show that cells isolated in 0.25 M sucrose, pH 7.3, retained the highest amount of lactate dehydrogenase activity in the cells. Cells prepared in 0.25 M sucrose, 20 mM Tris-HCl, retained more lactate dehydrogenase than cells prepared in 0.25 M sucrose 20 mM phosphate, pH 7.3. It was possible that the retention of lactate dehydrogenase in the cells was a function of the ionic strength of the dispersion media since Tris or phosphate buffer determines the ionic strength of dispersion media. Furthermore, phosphate has a higher ionic strength than Tris at comparable molarities. The effect of ionic strength on the retention of lactate dehydrogenase was examined with various concentrations of KCl in the dispersion medium. These results are presented in Table VI and show that the retention of lactate dehydrogenase in the cells was dependent upon the concentration

TABLE V

AVERAGE VALUES OF THE RETENTION OF THREE SOLUBLE ENZYMES
IN RAT LIVER CELL SUSPENSIONS PREPARED
IN 0.25 $\underline{M}$ SUCROSE, pH 7.3

Enzymes	No. of Exp.	Super-natant	Wash-I	Wash-II	Washed Cells
Lactate Dehydrogenase	16	53.1	5.0	1.7	40.2
3-Hydroxyanthranilate Oxygenase	3	78.3	16.0	3.5	2.2
Tyrosine Transaminase	2	80.8	12.5	2.8	3.9

TABLE VI

DISTRIBUTION OF LACTATE DEHYDROGENASE IN THE SUPERNATANT,
TWO WASHES AND CELLS FROM RAT LIVER CELL SUSPENSIONS
IN 0.25 $\underline{M}$ SUCROSE, pH 7.3 WITH VARIOUS
CONCENTRATION OF KCl

Experiment	KCl m$\underline{M}$	Cell Suspensions	Super-natant	Wash-I	Wash-II	Washed Cells
	0	100	37.1	6.3	2.4	54.2
	1	100	33.7	4.7	1.3	60.3
I	5	100	43.7	5.5	1.5	49.3
	10	100	--	--	--	--
	20	100	70.5	17.7	4.6	7.2
	50	100	--	--	--	--
	0	100	44.3	10.1	2.1	43.5
	1	100	--	--	--	--
II	5	100	57.2	5.4	1.6	35.8
	10	100	63.3	7.5	1.9	27.3
	20	100	75.6	12.5	3.4	8.5
	50	100	83.8	13.9	1.4	1.9

of KCl in the dispersion medium. The presence of 20 m$\underline{M}$ KCl in the medium reduced the retention of lactate dehydrogenase to that found with 20 m$\underline{M}$ Tris-HCl. Experiment II in Table VI clearly shows that the retention of lactate dehydrogenase was progressively reduced as the concentration of KCl was increased. These data support the view that the retention of lactate dehydrogenase in the rat liver cell suspension is a function of ionic strength of the dispersion media.

CHAPTER V

DISCUSSION

Efforts have been made to isolate intact parenchymal liver cells

since 1943. Up to present time no successful method of preparation

of intact parenchymal liver cells has been reported. In most prepara-

tion, microscopic examination of the cell suspensions showed that they

were homogeneous, well dispersed preparations of parenchymal cells.

However, there were large losses of various enzymes, especially those

of the soluble fraction of the cell,to the medium. Enzymes that were

almost completely lost to the medium include lactate dehydrogenase,

tryptophan pyrrolase, serine dehydrase and threonine dehydrase (5).

The present attempts to find a preparation procedure for isolation

of intact liver cells resulted in cells which retain lactate dehydro-

genase. The data presented in Table IV shows that lactate dehydrogen-

ase was retained in the isolated liver cells (40%) when buffers were

omitted from the dispersion media. This suggests that the retention

is dependent upon the ionic strength of the media. When the isolation

medium was 0.25 $\underline{M}$ sucrose, pH 7.3, lactate dehydrogenase was retained

in the cells (40%), whereas 3-hydroxyanthranilate oxygenase (2.1%)

and tyrosine transaminase (3.9%) were lost to medium as shown in Table

V. Increasing the concentration of KCl in the 0.25 $\underline{M}$ sucrose disper-

sion medium resulted in a progressive decrease in the retention of

lactate dehydrogenase in the isolated liver cells. These data suggest

that the retention of lactate dehydrogenase in isolated liver cells
is related to the ionic strength of the medium. Lactate dehydrogenase
exists as a tetramer of the mixture of M type (muscle) and H type
(heart) and adult rat liver contains mainly M_4 type (46). The fact
that low concentration of KCl added to the dispersion medium caused
a release of lactate dehydrogenase to the medium would suggest that
when cells are isolated in the presence of 0.25 M sucrose, lactate
dehydrogenase may be bound in some manner to the particulate portion
of the cell. The addition of KCl or other salts of comparable ionic
strength appears to result in dissociation and loss of lactate
dehydrogenase to the medium. These results would suggest that lactate
dehydrogenase in normal rat liver is associated with the particulate
portion of the cell, probably by some type of ionic linkage.

Takeda et al. (5) have observed that when rats were given gluco-
corticoids for several days prior to preparation of isolated liver
cells, these cells had an increased retention of lactate dehydrogenase
(up to 40%). However, the leakage of tryptophan pyrrolase, serine
dehydrase and threonine dehydrase was not prevented by prior treatment
of rats with glucocorticoids. Again, lactate dehydrogenase was the
only soluble enzyme that was retained. The glucocorticoids may in-
fluence the permeability of the cell membrane, thereby release other
soluble enzymes but retaining lactate dehydrogenase

In the presence of succinate the oxygen uptake of the cell sus-
pension was linear for about 60 minutes as shown in Figure 1. Addition
of glucose caused no significant alteration in the rate of oxygen
uptake. The failure of glucose to raise the oxygen consumption above

the endogenous level can be explained by the loss of the glycolytic

enzymes to the medium (16). When the vital stain, 1% Eosin Y (47),

was tested on the rat liver cell suspensions, only few cells could

exclude Eosin stain which indicated that most of the cells had damaged

membranes.

A tryptic digestion of liver was tried. 0.5 ml of trypsin solu-

tion was injected intravenously into the tail vein of rat and 15

minutes later, liver cell suspensions were prepared using routine

procedures. The isolated liver cells were similar to cells prepared

without trypsin with respect to the enzymatic leakage.

Recently Howard and Pesch (22) reported an enzymatic technique,

using collagenase and hyaluronidase, for the preparation of isolated

parenchymal cells from rat liver. Rat liver cell suspensions were

prepared from liver by incubation with 0.05% collagenase and 0.10%

hyaluronidase dissolved in calcium-free Hanks solution. The cells

were round and compact and had a well defined outline suggesting un-

damaged membranes. The isolated rat liver cells were far superior to

cells prepared by other methods since they excluded a vital stain and

had high endogenous respiration rates. However, the retention of

soluble enzymes was not checked.

In rats, tyrosine transaminase activity may be stimulated 3 to

4 fold by the administration of cortisol, and by immunological techni-

ques it has been shown that the increase in enzymatic activity is due

to the induction of specific enzymatic protein (48). Efforts to in-

duce tyrosine transaminase in rat liver cell suspensions by addition

of cortisol and RNA were made (49), but both cortisol and RNA failed

to increase the activity of tyrosine transaminase in the rat liver cell suspensions prepared by the present procedure.

The induction of certain liver enzymes, for example, tyrosine transaminase, may be mediated by glucocorticoids at the gene level. Cortisol was bound to various portions of the cells, even though tyrosine transaminase was not induced by cortisol. The failure to observe enzyme induction suggests that the cells as isolated do not exhibit normal liver function and this is probably due to the loss of soluble components from the cells. The binding of cortisol by isolated liver cells resembled the results of Ketchel and Garabedian (33) who examined the in vitro binding of cortisol by human leucocytes. They found that about 4% of cortisol was adsorbed from the medium and that the binding apparently followed a simple diffusion process since the rats of binding was directly proportional to the concentration of cortisol in the medium. Bellamy et al. (31) showed that in rat liver slices cortisol entered the cell by simple diffusion and a favorable gradient was maintained by intracellular absorption. In rat liver slices, about 85% of the intracellular cortisol was associated with the particulate fractions. In our studies on rat liver cell suspen-sions, 75% of the cortisol in the cells was associated with the parti-culate fractions. The nuclear fraction adsorbed slightly more cortisol than other fractions. This is interesting since it has been suggested that cortisol may mediate enzyme protein synthesis at the gene level.

Tomkins et al. (36) in 1966 investigated the induction of tyrosine transaminase by glucocorticoids in hepatoma tissue culture cells. Although this hepatoma tissue culture cell is one of the tumor culture

cell lines, it is minimally deviated from normal liver cells. That is, it closely resembles a normal liver cell. Most recently, Huang, in this laboratory prepared rat liver cell suspensions by the method of Howard and Pesch (22) and has shown that the activity of tyrosine transaminase in rat liver cell suspensions increase up to 10 fold by dexamethasone (50), a synthetic phosphoglucocorticoid.

At the present time, the use of isolated liver cells for the study of liver functions appears to be limited to particulate enzymatic systems. Because soluble cellular components leak out from the isolated cells, studies on soluble enzymatic systems are limited until an isolation procedure which results in cells with normal cell membrane characteristics is developed. It would appear that the enzymatic procedure of Howard and Pesch (22) results in intact parenchymal cells which have some degree of normal liver function. However, further studies are necessary to determine whether these cells can be used to study liver function under in vitro conditions. The recent findings of Huang (50) is the first example where isolated normal liver cells increase the activity of an enzyme in the presence of a hormone.

SUMMARY

During efforts made to find a preparation procedure for the
isolation of intact rat liver cells, it was found that lactate
dehydrogenase was retained in the isolated cells under certain con-
ditions. The retention was a function of the ionic strength of the
medium. Lactate dehydrogenase remained in the cells prepared in a
medium of very low ionic strength but was lost from the cells when
the ionic strength was increased in the medium. Under conditions
where lactate dehydrogenase was retained in the isolated cells, other
soluble enzymes such as tyrosine transaminase and 3-hydroxyanthranilate
oxygenase leaked out from the cells to the medium.

Rat liver cell suspensions incorporated about 4 to 5% of the
cortisol from the medium. The uptake of cortisol by the cells reached
a maximum by 20 minutes. There was a linear relationship between the
cortisol uptake and concentration in the range from 1 to 10 μg of
cortisol per ml of medium. Cortisol uptake was also in proportion to
the amount of cells in medium over an 8 fold range of cell concentra-
tion when the cortisol concentration was held at 0.7 μg/ml of medium.
Subcellular distribution studies showed that 75% of cortisol in the
cells was associated with the particulate fractions.

PART II

STABILIZATION, PURIFICATION AND PROPERTIES OF

BOVINE MAMMARY UDP-GALACTOSE 4-EPIMERASE

PART II

CHAPTER I

INTRODUCTION

Uridine diphosphogalactose 4-epimerase (UDP-galactose 4-epimerase)
(EC 5.1.3.2), an enzyme first described by Leloir (51) in galactose
adapted yeast, catalyzes the reversible transformation of uridine
diphosphogalactose (UDP-galactose)[*] into uridine diphosphoglucose.
This reaction is believed to be essential for the direct interconver-
sion of galactose and glucose in mammalian systems (52) as well as in
yeast (53), certain bacteria (54), and higher plants (55).

In 1937, Kosterlitz (56) started the pioneering work on galactose
metabolism. Kosterlitz found that the administration of large amounts
of galactose to rabbits gave rise to the accumulation of a hexose
ester which was identified two years later as α-galactose-1-phosphate.
During the next ten years rather little development occurred in this
field until Leloir and his coworkers started their important work on
galactose metabolism in yeast in 1948.

Leloir and his coworkers (57) were first to describe and purify
galactokinase which catalyzes the phosphorylation of galactose by

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[*]All abbreviations are in accordance with the IUPAC-IUB combined
Commission on Biochemical Nomenclature. J. Biol. Chem., 241, 527
(1966).

adenosine triphosphate (ATP) as shown in Reaction A.

A. Galactose + ATP $\longrightarrow$ Gal-1-P + ADP

In 1950, they discovered that a heat stable compound, identified as UDP-glucose, was essential for the enzymatic conversion of galac-tose-1-phosphate (Gal-1-P) to glucose-1-phosphate (Glc-1-P) (53). Again, in 1951 they demonstrated the existence of an enzymatic equili-brium reaction which occurs when UDP-glucose is incubated with dialyzed juice from galactose adapted yeast. The equilibrium mixture contains 75% UDP-glucose and 25% UDP-galactose (51). Leloir (51) postulated that the conversion of Gal-1-P to Glc-1-P occurs by way of the follow-ing two steps:

B. Gal-1-P + UDP-glucose $\underset{\text{transferase}}{\overset{\text{Gal-1-P uridyl}}{\rightleftharpoons}}$ Glc-1-P + UDP-galactose

C. UDP-galactose $\overset{\text{UDP-galactose 4-epimerase}}{\rightleftharpoons}$ UDP-glucose

Sum. B + C: Gal-1-P $\rightleftharpoons$ Glc-1-P
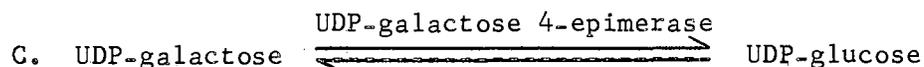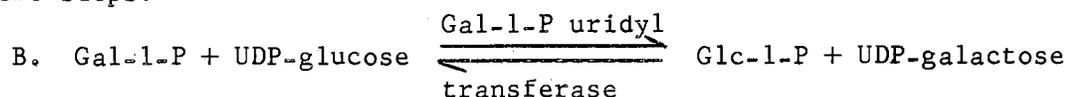
The term galactowaldenase was used to designate the overall enzymatic interconversion of Gal-1-P and Glc-1-P. Subsequently, the enzyme that catalyzes Reaction B, galactose-1-phosphate uridyl trans-ferase (EC 2.7.7.12), was demonstrated by Kalckar and his coworkers (58) in galactose adapted yeast and later in mammalian tissues (59) and bacteria (60). In order to emphasize the participation of two distinct and separable enzymes in the conversion of Gal-1-P to Glc-1-P, the enzyme that catalyzes Reaction C which was discovered by Leloir in 1951 has been designated UDP-galactose 4-epimerase by Kalckar since 1956 (61). Now, it is clear that galactose is converted by three reactions, A, B, C, to glucose-1-phosphate which is used for energy by hexose

phosphate pathways.

UDP-galactose 4-epimerase is important for the utilization of galactose as an energy source by way of the hexose phosphate pathways as mentioned above. It is also necessary for the synthesis of the galactose moiety of complex biological materials such as lactose, galactolipids and the blood group substances which are derived from glucose or its precursors.

It is believed that the synthesis of lactose in the mammary gland occurs via the following series of reactions (62).

$$\text{Glc-1-P} + \text{UTP} \underset{\xleftarrow{\hspace{4cm}}}{\overset{\text{UDP-glucose pyrophosphorylase}}{\xrightarrow{\hspace{4cm}}}} \text{UDP-glucose} + \text{P-P}$$

$$\text{UDP-glucose} \underset{\xleftarrow{\hspace{4cm}}}{\overset{\text{UDP-galactose 4-epimerase}}{\xrightarrow{\hspace{4cm}}}} \text{UDP-galactose}$$

$$\text{UDP-galactose} + \text{Glucose} \xrightarrow{\hspace{3cm}} \text{Lactose} + \text{UDP}$$
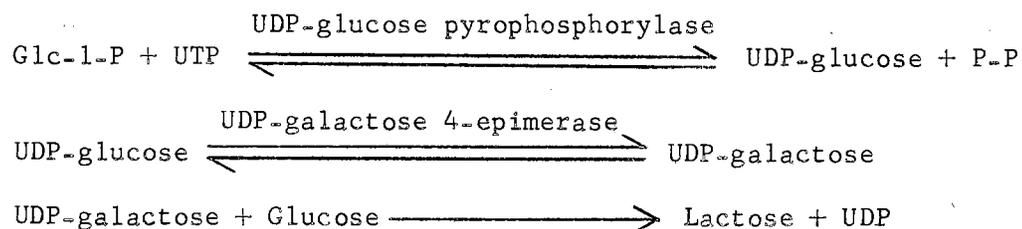
The activities of the enzymes involved in the synthesis of lactose are very low or absent in unstimulated mammary glands. The enzymatic activities increase somewhat during pregnancy, reach their highest level during lactation, and drop rapidly after weaning. The activity of UDP-galactose 4-epimerase in rat mammary glands shows a 10 fold increase in late pregnancy. Its activity rises dramatically in early lactation and reaches a level during late lactation which on the average is more than 300 times that of the unstimulated mammary gland (63). It is most probable that UDP-galactose 4-epimerase and other enzyme responsible for lactose biosynthesis are under homonal control.

In mammals, the peculiar brain galactolipids which are predominant in the myelinated substance of the nervous system are deposited during the first year after birth. In brain, UDP-galactose is a precursor of

galactolipids. With a microsomal preparation of rat brain and UDP-^{14}C-galactose as substrate, galactose could be incorporated into the lipid fraction (64). Another system for the synthesis of galactosyl-sphingosine was found in microsomes of guinea pig brain (65).

$$\text{UDP-galactose} + \text{Sphingosine} \rightleftharpoons \text{Galactosylsphingosine} + \text{UDP}$$

Galactose is present in certain immunochemically active substances such as the blood groups and lung galactan. The snail Helix pomatia produces highly branched polygalactosides in its mucus and the conch busycon also produces polygalactan. UDP-galactose might serve as the galactosyl donor in the biosynthesis of these polysaccharides (52).

Preliminary work on the purification of bovine mammary UDP-galactose 4-epimerase was initiated in this laboratory (66). As initially isolated, bovine mammary UDP-galactose 4-epimerase is a labile enzyme. The investigations described in second part of this dissertation will report on the stabilization, purification, kinetic studies and some properties of this enzyme. The chemical synthesis of the substrate UDP-galactose and a new compound, uridine diphospho-uridine (UDPU), which is an inhibitor of the enzyme, is also described.

CHAPTER II

LITERATURE REVIEW

Epimerase Reactions

According to the classification of the International Enzyme
Commission, epimerases belong to the fifth class "Isomerase" and its
first subclass "Racemase and Epimerase". Isomerases catalyzing
inversion of asymmetric groups are termed racemases or epimerases,
depending upon whether the substrate contains one or more centers of
asymmetry. Epimerization can be defined as the selective inversion
of configuration at single asymmetric center occuring in a compound
containing more than one such center (67). This type of reaction is
catalyzed in living systems by a group of enzymes called epimerases.
Epimerases are of interest not only because of their roles in metabolic
pathways but also because of the mechanisms by which the reactions
proceed.

Epimerization reactions are found in a number of carbohydrate
metabolic pathways. UDP-galactose 4-epimerase has been found in
mammalian systems as well as in yeast, certain bacteria and higher
plants (67). UDP-L-arabinose-4-epimerase (EC 5.1.3.5), which catalyzes
the interconversion of UDP-L-arabinose and UDP-D-xylose, was demons-
trated in plants by Neufeld et al. (68). Later Neufeld et al. (69)
showed that UDP-galacturonate-4-epimerase (EC 5.1.3.6), which catalyzes

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the reversible conversion of UDP-D-galacturonate and UDP-D-glucuronate, existed in mung bean seedlings. UDP-D-galacturonate-4-epimerase activity was also found in a strain of Pneumococcus type I whose capsular polysaccharide contains galacturonic acid (70). The enzymatic epimerization of UDP-N-acetyl-glucosamine to UDP-N-acetyl-galactosamine was found in extracts from calf liver (71). UDP-N-acetyl-glucosamine-4-epimerase (EC 5.1.3.7) was also found in the extract of a strain of Bacillus subtilis (72). Jacobson and Davidson (73) have found UDP-D-glucuronate-5-epimerase, which converts UDP-glucuronate to UDP-iduronate, in rabbit skin.

The epimerization of pentose phosphates is important in pentose metabolism. D-ribulose-5-phosphate-3-epimerase (EC 5.1.3.1), the enzyme that catalyzes the interconversion of D-ribulose-5-phosphate and D-xylulose-5-phosphate, has been demonstrated in mammalian tissues as well as in plants and bacteria (67). L-ribulose-5-phosphate-4-epimerase (EC 5.1.3.4) which catalyzes the conversion of L-ribulose-5-phosphate to D-xylulose-5-phosphate has been crystallized from E. coli (74).

Hydroxproline-2-epimerase (EC 5.1.1.8) was found in bacteria (75). N-acyl-D-glucosamine-6-phosphate-2-epimerase has been purified from bacteria (76). A similar enzyme, N-acyl-D-glucosamine-2-epimerase which catalyzes the interconversion of N-acyl-D-glucosamine and N-acyl-D-mannosamine has been found in animal tissues (77). Aldose-1-epimerase (EC 5.1.3.3), an enzyme which catalyzes the interconversion of the α and β anomers of glucose, has been purified from Penicillum notatum (78).

Isolation and Purification of UDP-Galactose
4-Epimerase from Various Sources

UDP-galactose 4-epimerase was partially purified from an extract of calf liver acetone powder (79). Wilson and Hogness (80) reported on the purification of UDP-galactose 4-epimerase from E. coli K 12 gal[+] (λdg). The purified enzyme from E. coli contained less than 10% impurity. Maxwell et al. (81) partially purified the enzyme from galactose adapted Saccharomyces fragilis. Recently, Darrow and Rodstrom (82) reported on the highly purified enzyme from yeast. Hansen and Craine (83) showed the presence of UDP-galactose 4-epimerase in Lactobacillus bulgaricus and purified it 15 fold from an extract of the bacterium. The purification of UDP-galactose 4-epimerase from cow's mammary acetone powder was initiated by Holmberg (66) and resulted in a 20 to 30 fold purification from the extract of an acetone powder.

The enzyme has not been crystallized nor has it been purified as a homogeneous protein from any source.

Stability of UDP-Galactose 4-Epimerase

The enzyme was stable during the process of purification from calf liver acetone powder (79), Saccharomyces fragilis (81), E. coli (80) and Lactobacillus bulgaricus (83). The purified calf liver enzyme could be kept frozen in 0.25 M glycylglycine at pH 7.5 for about a week without significant loss of activity. The lyophilized powder stored at -20°C was stable for at least three months (79).

The 150 fold purified yeast enzyme could be stored at -20°C, either as a solution in glycylglycine buffer or as a lyophilized powder, for at least four months (81). However, the stability of the further purified yeast enzyme (90 - 95% purity) varied. The highly purified yeast enzyme was kept frozen at -90°C. The preparation with the highest specific activity lost one third to one half of its activity within one week. The preparation with lower specific activities was more stable. More than 80% of the activity was lost by freeze drying. Storage for longer than 24 hours at 4°C at neutral pH resulted in a 50 to 90% loss in activity which was inversely proportional to the protein concentration between 0.5 and 10 mg/ml. However, up to 50% increase in activity were observed in some cases under the same conditions (82). Cations had no effect on the stability of the enzyme, although it is known that their presence greatly stimulated the rate of the epimerase reaction (84). 0.1% serum albumin was used to protect the diluted enzyme. Mercaptoethanol (7mM) could partially substitute for serum albumin but had no additional effect in the presence of serum albumin(83).

The purified enzyme from E. coli lost no activity when held at 4°C for at least a year at a concentration of 6.2 mg per ml. Variable results were obtained when the enzyme was frozen and stored at -20°C. In some cases one third of the activity was lost but in other cases all activity remained for one month (85). The enzyme from an extract of Lactobacillus bulgaricus was found to be stable at pH 5 at 4°C (83).

Specificity of UDP-Galactose 4-Epimerase

UDP-galactose 4-epimerase from yeast appeared to have a strict specificity for UDP-galactose or UDP-glucose. Three substrate analogs, UDP-D-xylose, UDP-P-L-arabinose and UDP-D-fucose, were epimerized by the partially purified yeast enzyme. However, further studies suggested that these three analogs were epimerized by contaminating enzymes other than UDP-galactose 4-epimerase (94).

The partially purified calf liver enzyme could epimerize its substrate analogs, 5,6-dihydro-UDP-glucose, 2'-deoxy-UDP-glucose and 2-thio-UDP-glucose. The initial rates of the conversion of 5,6-dihydro-UDP-glucose, 2'deoxy-UDP-glucose and 2-thio-UDP-glucose were 70%, 42% and 30% that of UDP-glucose respectively (95).

Ankel and Maitra (96) reported that the partially purified _E. coli_ enzyme converted UDP-xylose to UDP-arabinose reversibly. They concluded that a single enzyme was responsible for both the UDP-galactose and UDP-xylose epimerization reaction. This is just contrary to the situation with the yeast enzyme.

NAD^+ and UDP-Galactose 4-Epimerase

NAD^+ has been shown to be a coenzyme for UDP-galactose 4-epimerase isolated from calf liver, bacteria and yeast. The most striking characteristic of the enzyme purified from calf liver is the requirement for exogenous NAD^+ for full activity. The crude extract of the liver enzyme is stimulated by added NAD^+ and the stimulation by NAD^+ increases as the enzyme is more purified. The purified enzyme has an absolute requirement for NAD^+. The NAD^+ requirement can not be

substituted by NADH, NADP$^+$ or by any other NAD$^+$ analogs. The enzyme
is strongly inhibited by NADH (79).

Unlike the liver enzyme, the purified yeast enzyme does not
require NAD$^+$ nor is it inhibited by NADH. However, there is a close
correlation between enzymatic activity and a blue fluorescent spectrum
compatible with that of protein bound NADH during the purification of
yeast enzyme. The purified yeast enzyme contains tightly bound NAD$^+$
which survives through the purification procedure.

The E. coli enzyme is similar to the yeast enzyme. NAD$^+$ is
tightly bound to the purified enzyme and the addition of NAD$^+$ to the
E. coli enzyme does not increase the rate of the enzymatic reaction
(80). Kowalsky and Koshland (87) have found that the crude dialyzed
preparation of the enzyme from L. bulgaricus has no requirement for
NAD$^+$. The presence of UDP-galactose 4-epimerase in human hemolyzates
and in the homogenates of L cells and Hela cells could not be detected
unless an exogenous supply of NAD$^+$ was added to the extracts (88, 89).

<center>Fluorescence of Yeast UDP-Galactose 4-Epimerase</center>

UDP-galactose 4-epimerase isolated from yeast induced by galactose
exhibits a blue fluorescence which has an excitation maximum of around
350 mμ and an emission maximum of 435 mμ. The fluorescent spectrum
resembled that of NADH. There was a close correlation between
enzymatic activity and the blue fluorescence. Treatment of the
purified yeast enzyme with p-chloromercuribenzoate (PCMB) caused a
disappearence of the fluorescence as well as its catalytic activity.
The catalytic activity, but not the fluorescence, could be partially

restored by removing PCMB with a sulfhydryl reagent and addition of excess NAD^+ (86).

The fluorescence of the yeast enzyme may be attributed either to bound NADH or to a related NAD^+ complex which has an absorption maximum at 340 mμ. The enzyme bound NAD^+ could be converted to NADH by means of borohydride in the presence of a small amounts of substrate. This borohydride treated enzyme increased fluorescence 20 - 30 fold but lost more than 90% of its catalytic activity (90).

Yeast epimerase contains bound NAD^+ as well as NADH. The native yeast enzyme largely contains NAD^+ but has a small fraction with NADH which produced the fluorescence. The addition of $10^{-2}\underline{M}$ D-galactose and $10^{-3}\underline{M}$ 5'-uridylic acid (5'-UMP) to the yeast enzyme transformed it gradually into a highly fluorescent epimerase containing NADH, under the same conditions, the optical rotatory dispersion and circular dichroism of the enzyme also changed gradually as a function of time (92).

Guanidine and urea caused a decrease in both fluorescence and catalytic activity of the enzyme. The decrease in fluorescence was proportional to the concentration of guanidine after 60 minutes. The enzymatic activity was much more sensitive to guanidine than decrease in fluorescence. One $\underline{M}$ guanidine reduced the fluorescence to 60% and activity to 7% of their initial values in two hours. No recovery of either activity or fluorescence of the enzyme after treatment with guanidine or urea was observed upon removal of the denaturant by either dilution or dialysis (82). The changes in fluorescence, optical rotatory dispersion and circular dichroism were due to a conforma-

tional change of the enzyme (92).

P-Chloromercuribenzoate (PCMB) and
UDP-Galactose 4-Epimerase

The purified calf liver enzyme was inhibited by PCMB, and at 2×10^{-6}M, 50% of the enzymatic activity was inhibited. This inhibition could be completely overcome by cysteine (79). The addition of PCMB to the purified yeast enzyme caused a disappearance of fluorescence and a complete loss of activity as mentioned previously. Activity, but not fluorescence, could be partially restored by the addition of excess cysteine and NAD^+ (81). Unlike the calf liver and yeast enzyme, the activity of the partially purified E. coli enzyme was unaffected by PCMB (93).

Molecular Weight of UDP-Galactose 4-Epimerase

The yeast enzyme has a molecular weight of 125,000 as calculated from the sedimentation coefficient. It is composed of two subunits of about 60,000 molecular weight (82). The molecular weight of the E. coli enzyme was reported to be 79,000 (80). The molecular weight of calf liver enzyme has not been reported.

Kinetic Parameters of UDP-Galactose 4-Epimerase
from Various Sources

UDP-galactose 4-epimerase purified from different sources has many similar properties in general. The kinetic and physical parameters of UDP-galactose 4-epimerases from calf liver, yeast and E. coli are summarized in Table XLI.

TABLE XII

KINETIC AND PHYSICAL PARAMETERS OF
UDP-GALACTOSE 4-EPIMERASE

Kinetic Parameters	Sources		
	Calf Liver (29)	Yeast (31)	E. coli (30)
Km UDP-Galactose	5×10^{-5} $\underline{M}$	1.1×10^{-4} $\underline{M}$(34)	1.6×10^{-4} $\underline{M}$
Km UDP-Glucose	9×10^{-5} $\underline{M}$	-----	1.0×10^{-3} $\underline{M}$(43)
Keq $\dfrac{\text{UDP-Glucose}}{\text{UDP-Galactose}}$	3	3	3.5
pH Optimum	8.0 - 9.6	8.0 - 9.6	8.0 - 8.5
Exogenous NAD^+ for Catalysis	Yes	No	No
NADH Inhibition	Yes	No	No
Molecular Weight	-----	125,000 (32)	79,000
PCMB Inhibition	Yes	Yes	No (43)

Mechanism of UDP-Galactose 4-Epimerase Reaction

The biological interconversion of D-galactose and D-glucose involves the uridine diphosphate hexose rather than the free hexose or hexose phosphate (53). The proposed reaction mechanisms for the inversion of configuration at carbon 4 of the hexose moiety of UDP-hexose are: (I) dehydration to give a double bond between C-4 and either of the adjacent carbon atoms, followed by hydration to the other epimer, (II) direct inversion at C-4 by hydroxide ion, (III) cleavage to 3 carbon or other fragments followed by recondensation of the fragment, (IV) ring closure to an inositol followed by ring opening at a different position, (V) oxidation of the secondary alcohol group at carbon 4 to a keto group, followed by reduction to the other epimer.

Experiments carried out by Anderson et al. (97) and by Kowalsky and Koshland (87) using the L. bulgaricus enzyme ruled out the dehydration-hydration (mechanism I) and direct inversion by hydroxide ion (mechanism II) as possible mechanisms. In these experiments the reaction was carried out in T_2O or $H_2{}^{18}O$. Neither tritium nor ^{18}O was incorporated into the hexose. Similar results with respect to tritium were obtained with both the purified calf liver (61) and yeast enzyme (81).

Mechanisms III and IV were shown to be inoperative by feeding rats with C-1 and C-2 labeled galactose-^{14}C, isolating the glucose formed, and locating the label in the glucose (98). Since the label remained in its original position, it was concluded that inositol was

not an intermediate and that the carbon chain was not ruptured.

The requirement of exogenous NAD^+ for catalysis of the liver enzyme and the presence of bound NAD^+ in the yeast and E. coli enzymes suggest strongly oxidation-reduction (mechanism V) as an operative mechanism. Wilson and Hogness (80) have postulated the following model.

$$NAD\text{-}E + S_1 \rightleftharpoons NAD\text{-}E - S_1 \rightleftharpoons NADH - E - Sox$$
$$\rightleftharpoons NAD - E - S_2 \rightleftharpoons NAD - E + S_2$$
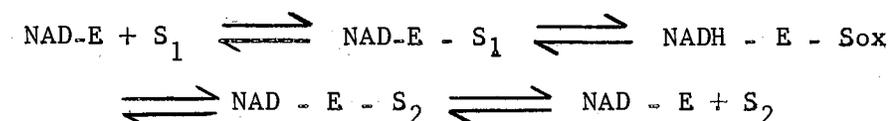
S_1 and S_2 in the above model represent the substrate UDP-glucose and UDP-galactose respectively. Sox is a hypothetical derivative of either UDP-hexose oxidized at carbon 4 of the hexose moiety, i.e., UDP-4-keto-glucose. NAD-E is the enzyme with bound NAD^+. Attempts to trap the 4-keto intermediate by carbonyl reagents such as thio-semicarbazide, hydrazine and hydroxylamine were unsuccessful. Further-more, tritium was not incorporated into the hexose from either NAD^+ labeled in the para position or NADH labeled in both para hydrogens. Thus, if NAD^+, reduced in the para position, is an intermediate in the reaction, it apparently does not exchange with exogenous labeled NADH (79). Budowsky et al. (95) proposed a hypothesis that uridine diphosphate hexose might form in solution a folded conformation due to hydrogen bonding between the uracil and hexosyl residues. The formation of this proper conformation of UDP-hexose was necessary for the reactivity of UDP-hexose in UDP-galactose 4-epimerase reaction. de Robinchon-Szulmajster (99) also postulated a molecular configura-tion of UDP-hexose similar to that of Budowsky (95) and explained the mechanism of oxidation-reduction (mechanism V) by a different approach.

Her mechanism of the epimerization reaction was based on two hydrogen aceptors (also serving as hydrogen donors), the uracil portion of the UDP-hexose and NAD^+. UDP-hexoses have a structure such that uracil could be in a position to accept one hydrogen from the hexosyl moiety while NAD^+ would accept the other hydrogen from the hexosyl moiety.

The mechanism by which the enzymatic epimerization takes place has not been elucidated. Most investigators would favor the hypothesis that the 4-keto hexose is an intermediate. The details of the reaction mechanism involving the role by NAD^+ as well as function of the uracil moiety of UDP-hexose remains to be determined.

CHAPTER III

MATERIALS AND METHODS

Materials

UDP-glucose, NAD$^+$, NADH, uracil, UMP, UDP, UTP, UDP-glucuronate,
AMP, ADP, ATP, CDP, GTP, PCMB, cysteine, glycine, glycylglycine,
bovine serum albumin, cytochrome C, lactate dehydrogenase, N,N'-
dicyclohexylcarbodiimide, galactose pentaacetate and snake venom were
purchased from Sigma Chemical Co., St. Louis, Missouri. Showdomycin
was obtained from P. L. Biochemicals, Inc.; iodoacetic acid from
Nutrional Biochemicals, Corp.; DEAE-cellulose (DE-32) from H. Reeve
Angel Inc.; morpholine from Calbiochem.;Bio Gel P-10 and Dowex 50
from Bio Rad Laboratories; Sephadex G-100 and blue dextran from
Pharmacia Fine Chemicals, Inc.; tributylamine and cyclohexamine from
Eastman Kodak Co.; and activated carbon from Barnebey-Cheney.
Nicotinamide mononucleotide and PRPP were obtained from Dr. R. K.
Gholson, Okla. State Univ. RNAase was a gift from Dr. F. R. Leach,
Okla. State Univ. All other chemicals were of reagent grade.

UDP-glucose dehydrogenase was purified from bovine calf liver
through Step V by the procedure of Strominger et al. (100). Alumina
gel C_r was made according to Methods in Enzymology (101). Hydroxyl-
apatite was prepared following the procedure of Siegelman et al.
(102) and could be reused once after washing with water and

58

equilibrating with the starting buffer.

Methods

Enzymatic Assay of UDP-Galactose 4-Epimerase

UDP-galactose 4-epimerase was assayed by two methods. Assay I, a direct spectrophotometric method adapted from the method of Maxwell et al. (79), was used for routine assays. Assay II, a two-step procedure adapted from the method of Imae et al. (93), was used in the studies on NAD^+ requirement, NADH inhibition, equilibrium constant and Km of UDP-glucose.

Assay I: The epimerase activity was measured spectrophotometrically by the rate of increase of absorbance at 340 mμ when UDP-galactose was present as the substrate. The reaction was coupled with an excess of UDP-glucose dehydrogenase and NAD^+.

$$UDP\text{-galactose} \rightleftharpoons UDP\text{-glucose}$$

$$UDP\text{-glucose} + 2\ NAD^+ \longrightarrow UDP\text{-glucuronate} + 2\ NADH + 2\ H^+$$
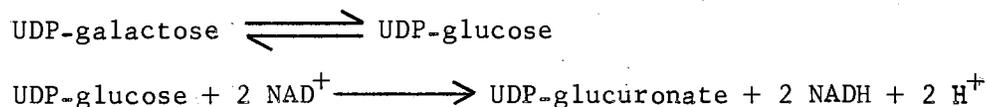
The reaction was carried out in a final volume of 0.5 ml of 0.1 $\underline{M}$ glycine buffer at pH 8.7 in a 0.75 ml cuvette. Each cuvette contained 50 μmoles of glycine, 0.5 μmoles of NAD^+, 0.2 μmoles of UDP-galactose, 400 units[*] of UDP-glucose dehydrogenase and a suitable amount of epimerase and deionized water to give a final volume of 0.5 ml. The epimerase samples were diluted in 25 m$\underline{M}$ potassium phosphate buffer at pH 7.6 containing 0.5 m$\underline{M}$ NAD^+ or UTP. The change in

----

[*]One unit of UDP-glucose dehydrogenase is defined as that of Strominger et al. (100).

absorbance at 340 mµ was measured by a Cary 14 spectrophotometer against a blank which contained all the above components except UDP-galactose. The rate of reaction was proportional to enzyme concentration up to a change in absorbance of 0.05 per minute as shown in Figure 6. One unit of enzyme was defined as that amount which catalyzed an increase in absorbance at 340 mµ of 0.001 per minute at 25°C under the condition described. One unit is equal to 4×10^{-5} units (µmoles/min.) defined by the International Union of Biochemistry.

It was reported that the activity of yeast epimerase was 10 times greater when glycine buffer was replaced by glycylglycine buffer (84). The activity of bovine mammary epimerase increased only 20% when glycylglycine buffer was used instead of glycine buffer under the assay conditions described above.

Assay II: Various amounts of enzyme purified through Step VII were incubated for 5 minutes at 25°C with 20 µmoles of glycine buffer at pH 8.7, 0.02 µmoles of NAD^+, 0.2 µmoles of UDP-galactose and various amounts of deionized water to give a final volume of 0.2 ml. The reaction was stopped by placing in boiling water for 3 minutes and the reaction mixture was immediately put into an ice bath. The UDP-glucose formed was measured with excess UDP-glucose dehydrogenase in a reaction mixture which contained 100 µmoles of glycine at pH 8.7, one µmole of NAD^+, 800 units of UDP-glucose dehydrogenase, 0.4 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 against a blank which contained all reagents except that the incubation mixture was replaced by deionized water. The UDP-
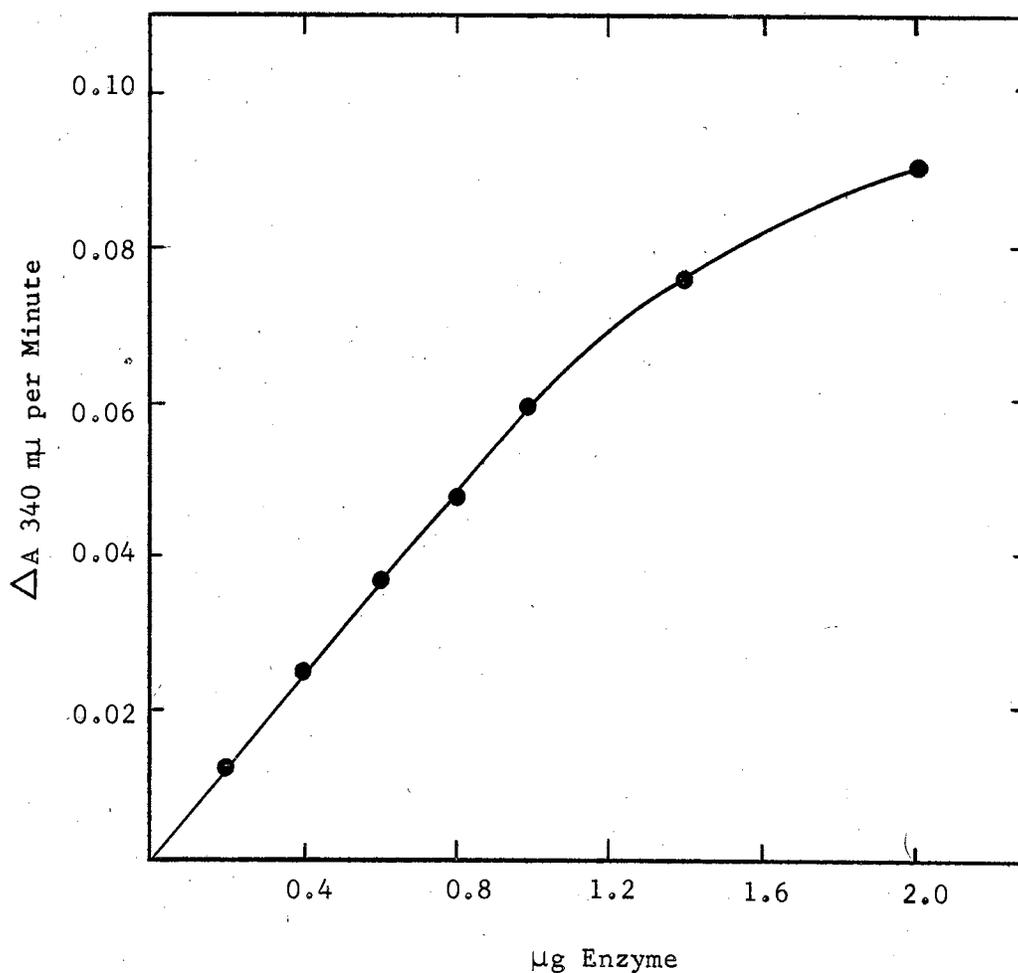
Figure 6. Linearity of Assay I with Respect to the Amount of UDP-Galactose 4-Epimerase.

Assay I was conducted with various amounts of the enzyme purified through Step VII and diluted with 20 mM potassium phosphate buffer which contained 0.5 mM NAD^+, pH 7.6. The reaction mixture contained 50 μmoles of glycine, pH 8.7, 0.5 μmoles of NAD^+, 0.2 μmoles of UDP-galactose and various amounts of the 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.

glucose formed was calculated from the amount of NADH produced.

The linear relationship between amount of enzyme and UDP-glucose formed during 5 minutes of incubation is shown in Figure 7. The relationship between time of incubation and amount of UDP-glucose formed under the assay conditions described above is shown in Figure 8.

Other Methods

DEAE-cellulose (DE-32) was prepared and regenerated by washing batchwise with 15 volumes of 0.5 N NaOH for 30 minutes, distilled water until neutral, 15 volumes of 0.5 N HCl for 30 minutes, distilled water until pH was between 4 and 5, 0.5 NaOH for 15 minutes and finally with distilled water until neutral. DEAE-cellulose obtained was equilibrated with 1 M appropriate buffer at the desired pH. The pH of the cellulose was measured by placing the electrode directly into the cellulose itself. Differences in the pH between the supernatant solution and the cellulose itself were observed. Just before use, DEAE-cellulose was washed with an appropriate buffer until the conductivity of the effluent solution was equal to that of the buffer.

Protein was routinely determined by the method of Lowry et al. (103). Conductivity was measured with a Radiometer conductivity meter. Fractions off columns were collected with a Beckman Model 32 fraction collector. Disc electrophoresis was run with a Canalco Model 66 electrophoresis apparatus. The enzymatic assay for lactate dehydrogenase (EC 1.1.1.27) was described in Part I.

μμoles UDP-Glucose Formed
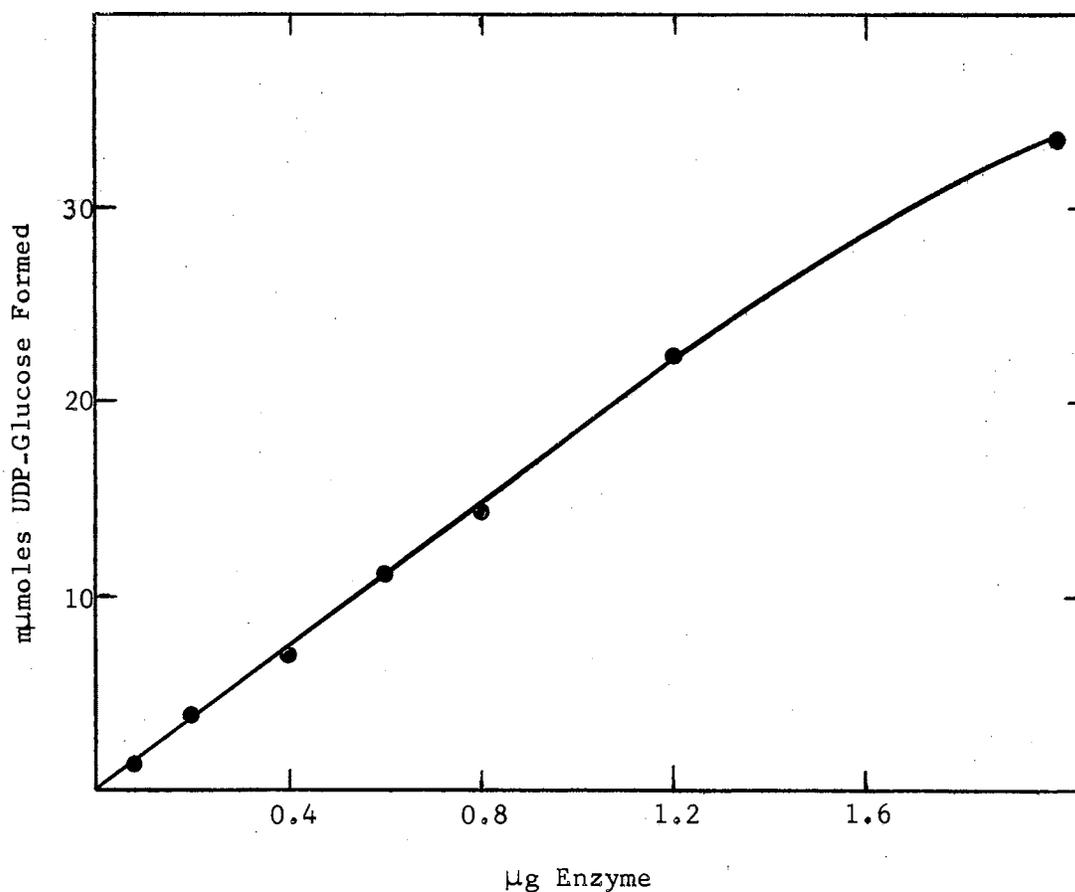
μg Enzyme

Figure 7. Relationship between the Amount of Epimerase and UDP-Glucose Formed by Assay II.

Assay II for UDP-galactose 4-epimerase was run with various amounts of the epimerase purified through Step VII. The enzyme was incubated for 5 minutes at 25°C with 20 μmoles of glycine buffer at pH 8.7, 0.02 μmoles of NAD$^+$, 0.2 μmoles of UDP-galactose and a sufficient amount of deionized water to give a final volume of 0.2 ml. The reaction was stopped in boiling water for 3 minutes and diluted with deionized water to 0.5 ml. The UDP-glucose formed was calculated from the total absorbance at 340 mμ in a reaction mixture containing 100 μmoles of glycine buffer, pH 8.7, one μmole of NAD$^+$, 800 units of UDP-glucose dehydrogenase, 0.4 ml of the above reaction mixture and water in a final volume of 1 ml.
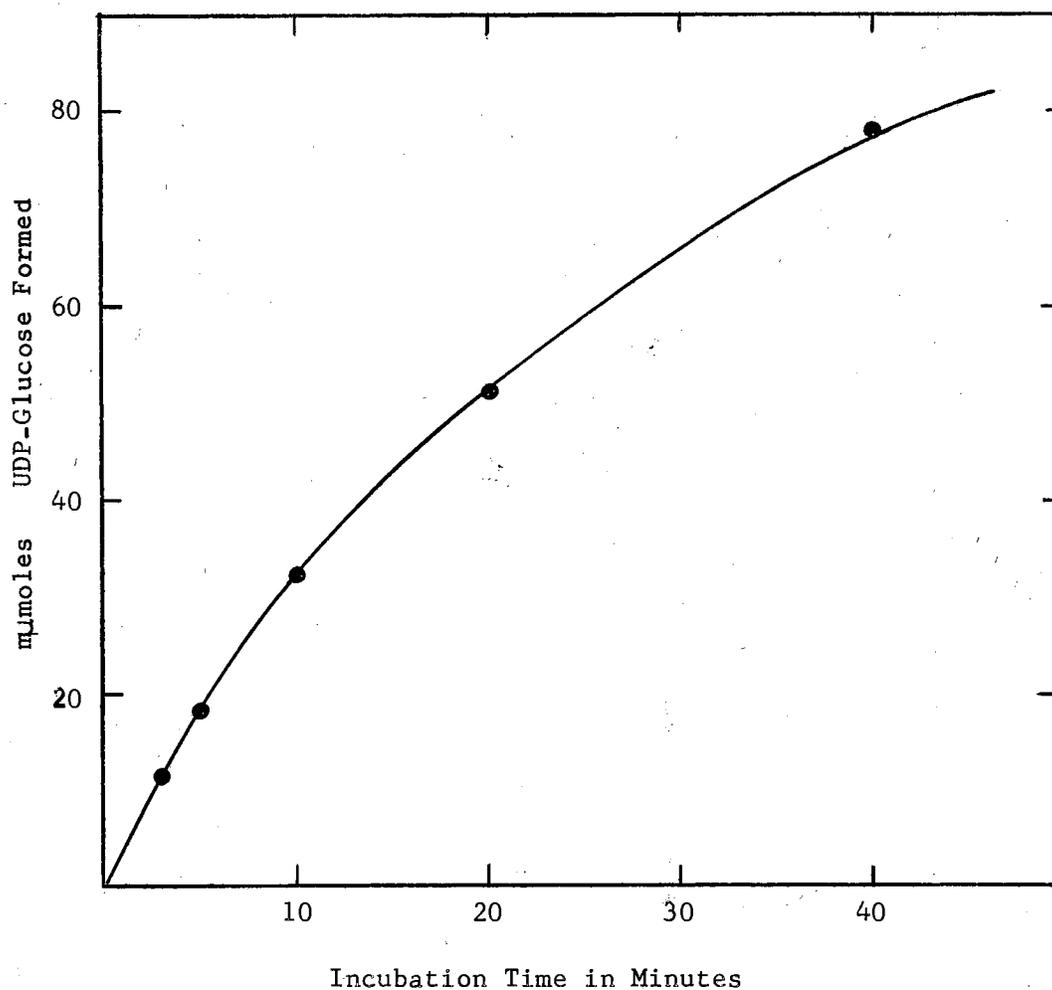
Figure 8. Relationship between the Time of Incubation and
the Amount of UDP-Glucose Formed by Assay II.

6 μg of the epimerase purified through Step VII were
incubated at 25°C in 2 ml of buffer containing 200 μmoles
of glycine at pH 8.7, 0.2 μmoles of NAD^+, 20 μmoles of UDP-
galactose. 0.2 ml of aliquots were taken out at incubation
times of 0, 3, 5, 10, 20, 40 minutes and the enzymatic reac-
tion was stopped in boiling water for 3 minutes. The ali-
quots of 0.2 ml were diluted with water to 0.5 ml. The
UDP-glucose formed was calculated from the total absorbance
at 340 mμ in a reaction mixture which contained 100 μmoles
of glycine buffer, pH 8.7, 1 μmole of NAD^+, 800 units of
UDP-glucose dehydrogenase, 0.4 ml of the above reaction
mixture, and water in a final volume of 1 ml.

CHAPTER IV

CHEMICAL SYNTHESIS OF UDP-GALACTOSE
AND URIDINEDIPHOSPHOURIDINE

Chemical Synthesis of UDP-Galactose

Preparation of Uridine-5'-Phosphoromorpholidate

A sample of UMP-morpholidate was prepared according to the method
of Moffatt and Khorana (104).

The disodium salt of UMP (0.6 g) was dissolved in 40 ml of de-
ionized water and passed through a Dowex 50 (H^+) column (2.5 x 25 cm).
The column was washed with water until the washings were neutral to
litmus paper. The solution was concentrated to 15 ml at 10°C on a
rotatory evaporator. This concentrated solution was then treated with
15 ml of t-butyl alcohol and 0.5 ml of redistilled morpholine. The
solution was heated at refluxing temperature, and while refluxing, a
solution of dicylohexylcarbodiimide (1.236 g) in 22 ml of t-butyl
alcohol was added dropwise over a three hour period. The mixture was
heated overnight and found to be free of starting nucleotide (UMP) as
detected by paper chromatography. The reaction mixture was cooled
and the colorless crystals were filtered out and washed with a little
t-butyl alcohol. The solvent was evaporated under diminished pressure
with a water pump. The filtrate was then treated with ether and
filtered to remove any solid. The aqueous solution was extracted

twice with ether and the ether solution was washed with water. The

aqueous solution and the washings were combined and concentrated first

on a water pump and then to complete dryness under vaccum using an oil

pump. The glassy solid thus obtained was dissolved in 4 ml of methanol

and transferred to a centrifuge tube and treated with anhydrous ether.

The gummy solid was triturated with fresh anhydrous ether and a white

solid separated out. It was filtered and dried in a desiccator over

magnesium perchlorate under vaccum. The UMP-morpholidate was obtained

as 0.6 g of the salt of 4-morpholine N,N'-dicyclohexylcarboxamidine

and was in the hydrated form.

Preparation of Tri-n-Butylammonium Salt of Gal-1-P

The cyclohexylammonium salt of Gal-1-P was prepared by the method

of Macdonald (105). D-galactose pentaacetate was selectively phos-

phorylated at the C-1 position by anhydrous phosphoric acid and the

acetyl groups were removed by alkaline hydrolysis. The Gal-1-P was

crystallized as the cyclohexylammonium salt.

The change of salt form from Gal-1-P cyclohexylammonium salt to

the tri-n-butylammonium salt was based on the procedure of Roseman

et al. (106) except that tri-n-butylamine was used instead of tri-n-

octylamine.

The cyclohexylammonium salt of Gal-1-P (0.9 g) was dissolved in

40 ml of deionized water and passed through a Dowex 50 (H^+) column

(2.5 x 20 cm). The column was washed thoroughly until the washings

were neutral. The total eluate was made alkaline by adding pyridine

and concentrated in vacuo to about 5 ml at 10°C. The concentrated

solution was then dissolved in 15 ml of pyridine and a solution of 1.44 ml of tri-n-butylamine in 15 ml of pyridine was added dropwise. The mixture was stirred until clear. The homogeneous solution was then evaporated to dryness and the residue was rendered anhydrous by dissolving it in anhydrous pyridine and evaporating the solvent in vacuo four times. Finally it was triturated with anhydrous ether and a white solid separated out. The ether was decanted and the product was dried in vacuo in a desiccator containing magnesium perchlorate.

Preparation of UDP-Galactose

A total of 2 grams of UDP-galactose (III) were synthesized using the method of Roseman et al. (106). The 4-morpholine N,N'-dicyclohexylcarboxamidinium salt of uridine-5'-phosphoromorpholidate (I) was reacted with tri-n-butylammonium salt of Gal-1-P (II).

(I) + (II)

(III)

R = uracil;   R' = D-galactose
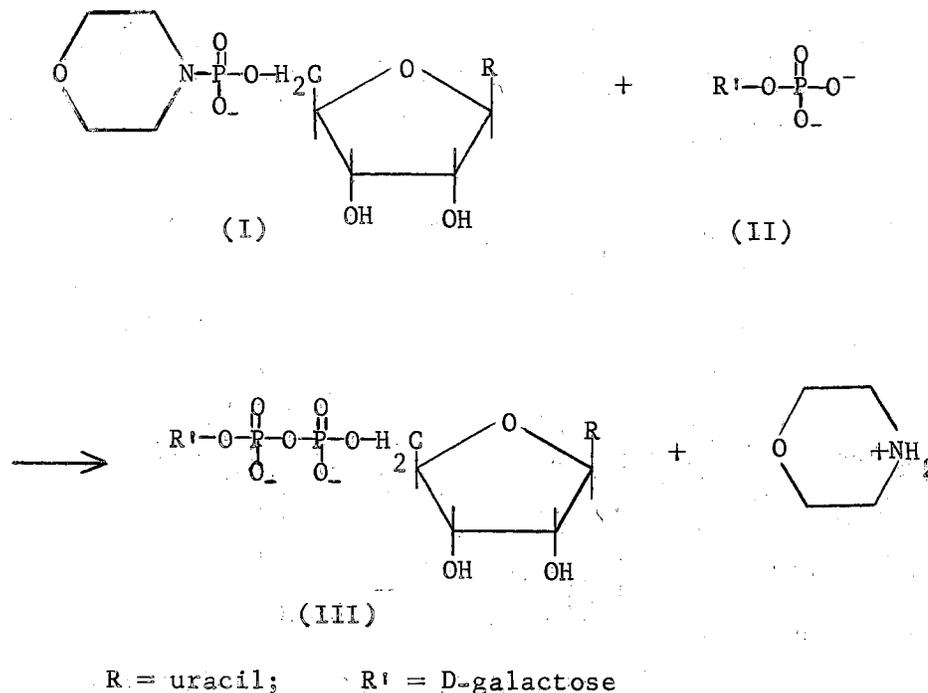
The tri-n-butylammonium salt of Gal-1-P (1.0 g) was dissolved in about 10 ml of anhydrous pyridine. The solution was added to 1.17 g of the 4-morpholine N,N'-dicyclohexylcarboxamidinum salt of UMP-morpholidate. The mixture was evaporated in vacuo to dryness. The residue was redissolved in 17 ml of anhydrous pyridine and kept at room temperature for 5 days. The reaction mixture was checked by paper chromatography for formation of UDP-galactose.

After pyridine was removed from reaction mixture in vacuo, UDP-galactose was separated out from other components by eluting from a DEAE-cellulose column (5 x 35 cm) with a linear gradient from 0 to 250 mM triethylamine-acetate at pH 5.5 using 2 l of water and 2 l of buffer. A 0.646 g sample of UDP-galactose disodium salt was obtained. The product showed only one spot on paper chromatography. The UDP-galactose prepared by this method was used in the routine assays of UDP-galactose 4-epimerase.

Chemical Synthesis of Uridinediphosphouridine

Attempts to synthesize UDP-4-ketoglucose, a hypothetical inter-mediate in UDP-galactose 4-epimerase reaction, were made by Dr. W. A. Khan in this laboratory. The method for the chemical synthesis of UDP-galactose (106) was tried for the synthesis of UDP-4-ketoglucose by using 4-ketoglucose-1-P instead of galactose-1-P. Because 4-ketoglucose-1-P is unstable during the reaction process, the synthesis of UDP-4-ketoglucose was unsuccessful. However, a small quantity of an unknown compound which strongly inhibits the UDP-galactose 4-epimerase reaction was obtained. This unknown compound was thought to

be uridinediphosphouridine (IV) (107). The synthesis of IV from the condensation of two molecules of UMP-morpholidate was tried and efforts to identify it were made.

Preparation of Uridinediphosphouridine

The formation of UDPU (IV) from UMP-morpholidate (I) may be explained either by a slow hydrolysis (absorption of moisture by pyridine) of UMP-morpholidate to UMP followed by anionic attack of UMP on UMP-morpholidate (Reaction A), or by the self condensation of the UMP-morpholidate to V followed by hydrolysis of V during work up (Reaction B).

A.

(UMP)   (I)

(IV)

R = uracil

B.   2   (I)

(V)
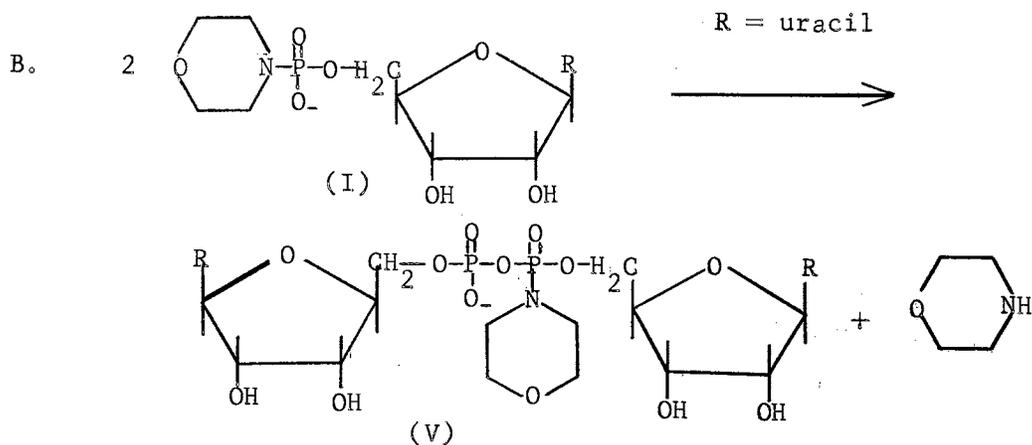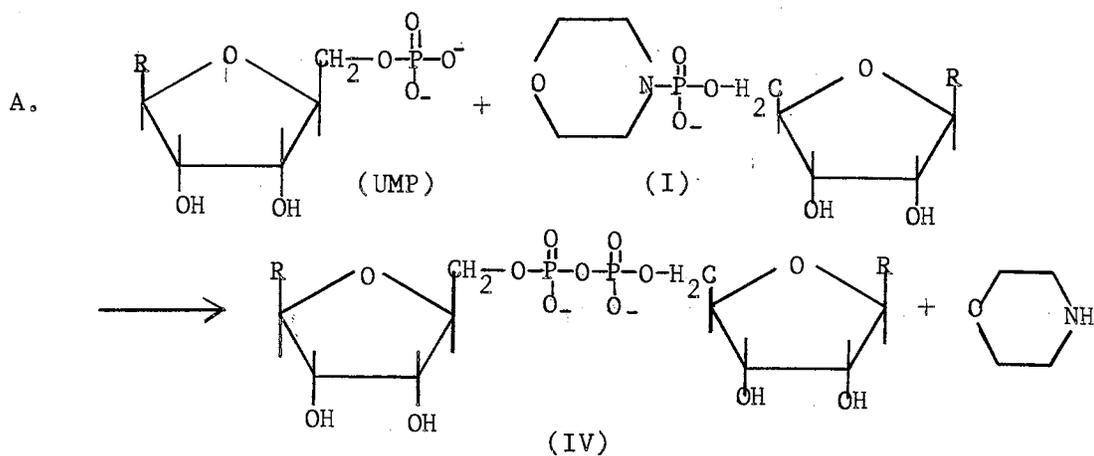
The 4-morpholine N,N'-dicyclohexylcarboxamidinium salt of UMP-
morpholidate (0.4 g) was dissolved in 7 ml of anhydrous pyridine and
the solution was kept at 70°C for 3 days. Pyridine was evaporated
from the reaction mixture in vacuo and the residue was shaken with a
mixture of water and ether containing 0.56 g of sodium acetate. The
aqueous layer was separated out and again extracted with ether and
ether layer was back washed with water. The aqueous solution was
diluted with water to reduce the conductivity to 0.4 m MHO and then
passed through a DEAE-cellulose column (3.0 x 3.5 cm) at pH 5.5. The
materials shown as peaks in Figure 9 were eluted from 0 - 250 mM of
triethylamine-acetate at pH 5.5 using 1.5 1 of water and 1.5 1 of
buffer to form the gradient solution. Fractions of 12.5 ml were
collected at a flow rate of 1 ml per minute. Fractions containing IV
were pooled and were evaporated to a syrup in vacuo. The residue was
treated with 10 ml portions of methanol and evaporated to dryness
(syrup) until the ordor of triethylamine was absent. The final residue
was dissolved in about 2 - 3 ml methanol, a 1 M solution of sodium
iodide in acetone (2 equivalents relative to UDPU by the absorbance
at 262 mμ) was added followed by about 100 ml of acetone. The result-
ing precipitate was collected by filtration, washed three times with
10 ml portions of acetone and dried in vacuo over magnesium per-
chlorate. The product weighed 52.5 mg.

Characterization of Uridinediphosphouridine

1. Paper Chromatography of Compound IV.

The preparation of IV showed one spot on paper chromatography.
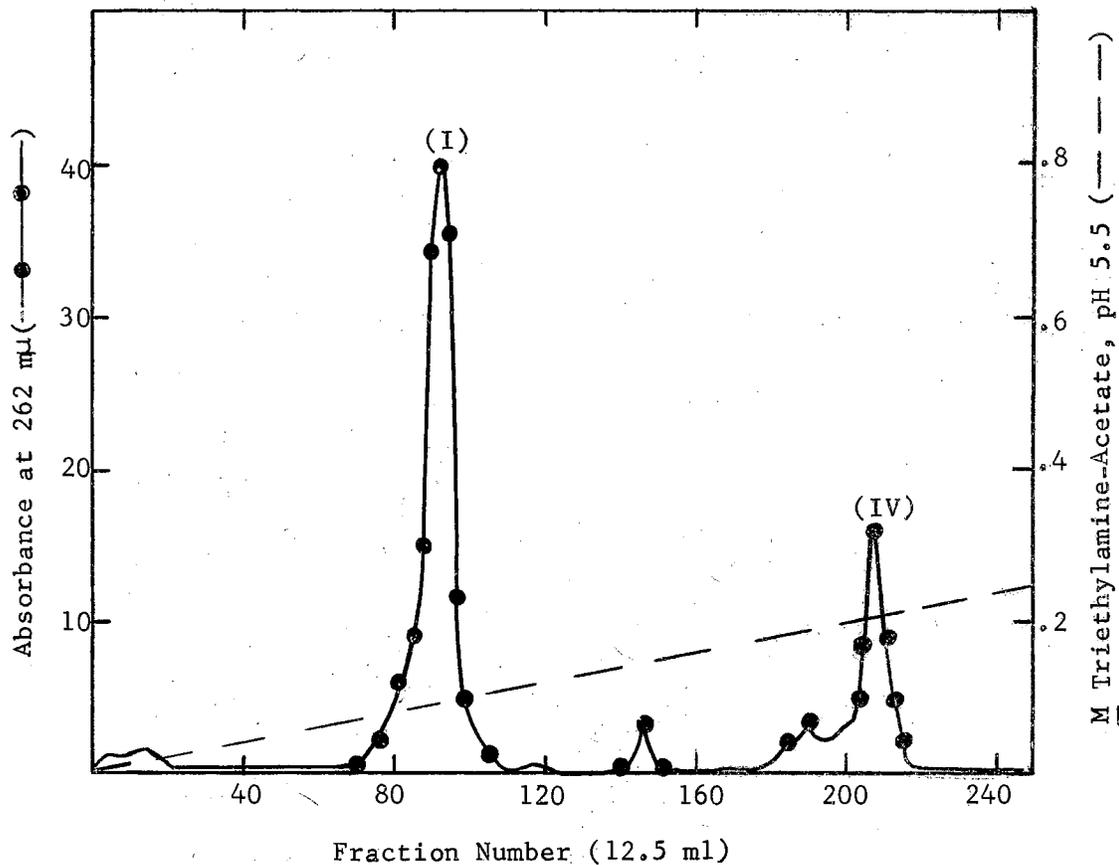
Figure 9.   Separation of Compound IV from the Reaction Mix-
ture by DEAE-Cellulose Chromatography.

The reaction mixture was put on a DEAE-cellulose column
(3.0 x 3.5 cm) and was eluted with a linear gradient from 0
to 250 mM triethylamine-acetate at pH 5.5 using 1.5 1 of
water and 1.5 1 of 250 mM triethylamine-acetate. Fractions
of 12.5 ml were collected at a flow rate of one ml per min.
The concentration of nucleotide was monitored by the absorbance
at 262 mμ on a Beckman DU spectrophotometer.

A descending paper chromatography was carried out with Whatman No. 1
paper using a solvent system of 95% ethanol : 1 M ammonium acetate at
pH 3.8 (75 : 30 v/v) (108) for 8 - 10 hours at room temperature.
After the paper was air dried, the ultraviolet absorbing materials
were detected in a Chromato-Vue at 254 mμ. If the detection of Pi
or compounds containing phosphate was desired, the paper was sprayed
with the Hanes-Isherwood reagent (109), Pi appeared immediately as a
yellow apot. If the paper was warmed to 85°C - 100°C for 3 - 5
minutes and then was exposed to ultraviolet radiation for 10 minutes,
organic phosphate compounds (acid labile) appeared as blue spots (110).

The Rf values of compound IV, UDPG, UDP, UMP, UMP-morpholidate,
uridine, Glc-1-P and Pi were as follows:

Compound	Compound IV	UDPG	UDP	UMP	UMP-morpholidate	Uridine	Glc-1-P	Pi
Rf	.17	.19	.21	.41	.58	.68	.37	.46

2. Acid Hydrolysis of Compound IV.

Uridinediphosphate, UDP-glucose and compound IV were subjected to
1 N HCl hydrolysis in a boiling water bath (92°C - 95°C) for various
periods of time. The hydrolyzed products were analyzed by paper
chromatography as previously described. At the 7th minute, most of
the UDP was hydrolyzed to UMP and Pi while some remained unchanged;
UDP-glucose to UMP and Pi leaving a trace of unhydrolyzed UDP-glucose.
IV showed two spots with about equal intensity as detected at 254 mμ,
one corresponding to IV and the other to UMP. UDP was completely
hydrolyzed to UMP and Pi in 30 minutes; UDP-glucose to UMP and Pi in
15 minutes. IV was hydrolyzed to only one spot corresponding to UMP
in 60 minutes.

3. Enzymatic Digestions with Snake Venom (Trimeresurus Flavoviridis)

and RNAase.

UDP-glucose and IV were incubated with snake venom at room

temperature for various lengths of time. The components in the incu-

bated mixture were analyzed with paper chromatography as mentioned

above. Part of the UDP-glucose was first hydrolyzed to uridine, Pi

and Glc-1-P, and finally all the UDP-glucose was hydrolyzed. Uridine

and Pi were first partly formed from IV, and finally all of IV was

degraded after prolonged incubation. No UMP was detected in both

cases. This is similar to RNA hydrolysis by crude snake venom which

contained diesterase and 5'-nucleotidase. Only nucleosides and Pi,

but no nucleotides, were found in the hydrolyzate (111).

RNAase cleaves the phosphodiester bond between 3' and 5' positions

of the ribose moiety in RNA. Compound IV remained unchanged when it

was incubated with RNAase for 4 hours. This indicates that IV does

not have a phosphodiester bond with a 3' and 5' linkage.

4. Ultraviolet Spectrum of Compound IV.

Compound IV was dissolved in water and its ultraviolet spectrum

was determined with a Cary 14 spectrophotometer from 215 mμ to 340 mμ.

The ultraviolet spectrum of IV was typical of uridine (112).

5. Estimation of the Molecular Weight of Compound IV.

An aliquot of 0.09 ml solution containing 0.4 mg per ml was

diluted with 0.91 ml of water in a 1.5 ml cuvette. The absorbance at

262 mμ was 0.98 as recorded on a Cary 14 spectrophotometer.

The equation, $A = E \cdot C \cdot 1$, was used to determine the molecular

weight where A is absorbance at 262 mμ, E is molar extinction

coefficient, C is the concentration (mole per liter), l is length of the cell (1 cm). The molar extinction coefficient of UMP is 10,000 (113). Assuming that IV contains two UMP residues, E for IV will be 20,000. The molecular weight of IV was calculated to be 735. The theoretical molecular weight for uridinediphosphouridine disodium salt dihydrate (UDPUNa$_2 \cdot$2H$_2$O) is 710.

From the results of acid hydrolysis, snake venom digestion, ultraviolet spectrum and molecular weight estimation, compound IV was identified as uridinediphosphouridine (UDPU).

CHAPTER V

PURIFICATION OF UDP-GALACTOSE 4-EPIMERASE
FROM BOVINE MAMMARY TISSUES

Lactating bovine mammary glands contain large amounts of fat

which makes the early steps of an enzymatic purification difficult.

Therefore, an acetone powder of bovine mammary tissue was used as the

starting material for the purification of UDP-galactose 4-epimerase.

The epimerase in the acetone powder is stable for 6 months when stored

at -20°C.

Acetone Powder of Mammary Tissue

Lactating mammary glands were obtained immediately after slaughter

from cows at the Wilson Packing Plant in Oklahoma City, Oklahoma.

The mammary tissue was cut into small pieces and frozen in powdered

dry ice. The frozen tissue was thawed overnight at 4°C and ground

in a heavy-duty mechanical meat grinder. The ground tissue, in 150 g

portions, was extracted with 10 volumes of acetone at -10°C by blend-

ing it for three periods of 20, 30 and 30 seconds respectively.

Temperature was maintained at about -10°C by the addition of powdered

dry ice. The blended material was filtered through shark skin filter

paper under suction while the temperature was kept at about -10°C by

sprinkling with powdered dry ice. The moist cake was immediately

blended again for three periods each of 20 seconds in 10 volumes of

acetone at -10°C as previously described. The blended material was filtered and pressed dry on the suction filter funnel. Then the cake was rubbed with the hands into small pieces or fine fibers until dry. The mammary tissue acetone powder was further air dried for 4 to 6 hours on brown paper at room temperature and then was stored at -20°C in a dessicator.

Preliminary Experiments

Composite buffer (0.2 $\underline{M}$ Tris, 10 $\underline{mM}$ MgCl$_2$, 1 $\underline{mM}$ EDTA, 1 $\underline{mM}$ mercaptoethanol, pH 7.6) was used to extract the enzyme from the mammary tissue acetone powder (16). Extraction of the enzyme with various kinds of buffers were examined and the results are shown in Table VIII. Among the buffers tested, 0.25 $\underline{M}$ potassium phosphate buffer at pH 7.6 had the highest activity per ml of buffer. The activity per ml of 0.25 $\underline{M}$ potassium phosphate buffer was twice that of the composite buffer, and the protein concentration of the extract of 0.25 $\underline{M}$ potassium phosphate buffer was only one half that of the extract of the composite buffer. Hence the specific activity of 0.25 $\underline{M}$ potassium extract is four times that of composite buffer extract. When the acetone powder was extracted with 0.25 $\underline{M}$ potassium phosphate buffer and portions of the extract were taken out, centrifuged and assayed for the enzymatic activity at periods of 30, 60, 90 and 120 minutes, the extract at 60 minutes gave the highest activity per ml. As the result, the extraction of the enzyme from the acetone powder was carried out with 0.25 $\underline{M}$ potassium phosphate buffer at pH 7.6 for 60 to 70 minutes.

TABLE VIII

EXTRACTION OF UDP-GALACTOSE 4-EPIMERASE FROM ACETONE
POWDER WITH VARIOUS KINDS OF BUFFER

Buffers	Enzymatic Activity Units per ml	Appearence of Supernatant
Distilled Water	4,600	Turbid
Composite Buffer	2,500	Turbid
5 mM Potassium Phosphate, 2 mM Mercaptoethanol, pH 7.6	4,400	Turbid
0.25 M Potassium Phosphate, pH 7.6	4,960	Clear
20 mM Tris, 5 mM $MgCl_2$, pH 7.6	4,160	Turbid
10 mM Sodium Acetate, 5 mM $MgCl_2$, pH 6.0	4,200	Turbid
0.1 M KCl, 50 mM Tris, pH 7.4	3,280	Turbid
0.15 M KCl, 15 mM Citrate, pH 7.4	4,800	Turbid

2 g of acetone powder was stirred with 30 ml of the different buffers for 60 minutes and then was centrifuged at 25,000 x g for 15 minutes. The supernatant solutions obtained were assayed for enzymatic activity.

Salt fractionations were examined next. A 30 - 60% ammonium sulfate fractionation of the extract resulted in a two fold purification. Refractionation of the 30 - 60% ammonium sulfate fraction with a 4 $\underline{M}$ K_2HPO_4 solution or a saturated alkaline ammonium sulfate solution increased the specific activity of the epimerase. A saturated alkaline ammonium sulfate solution was chosen as a fractionation agent for two reasons: (1) consistency of purification was observed, and (2) the epimerase was easily centrifuged out of the solution.

Organic solvent fractionation was tried on the 30 - 60% ammonium sulfate preparation with acetone or ethanol and was unsuccessful. pH fractionation was examined by adjusting the pH of the epimerase preparation to 9.8 by adding 1 N ammonium hydroxide, or to 5.5 by adding 1 N acetic acid. The addition of both acid and base caused a drastic loss of activity.

Both calcium phosphate gel and alumina C_r gel could adsorb non-enzymatic protein from the alkaline ammonium sulfate preparation. The calcium phosphate had a higher adsorbing capacity but an excess of the adsorbent was frequently committed and resulted in a low yield. Hence the less adsorbing alumina C_r gel was used to avoid this problem.

When a partially purified epimerase preparation was placed on a DEAE or hydroxylapatite column and eluted off the column, a tremendous loss of activity occurred. Unless the problem of stability of the epimerase was solved, the purification of the enzyme by column chromatography technique would be useless. Efforts were made to stabilize the epimerase. NAD^+ was first compound found able to stabilize a diluted enzymatic solution in which the epimerase is

extremely labile without the presence of NAD^+. Therefore, all purification involving column chromatography were done in the presence of 0.5 $\underline{mM}$ NAD^+. The stabilizing effect of NAD^+ and other agents on the epimerase will be documented in the next chapter.

Prolonged dialysis against 25 $\underline{mM}$ phosphate at pH 7.6 caused a loss of activity. Overnight dialysis of a concentrated epimerase preparation (20 - 30 mg/ml) lost about 50% of its activity. Incorporation of 1 $\underline{mM}$ NAD^+ with the epimerase sample during dialysis did not prevent the decay of the activity. Therefore, a short term dialysis (2 hours) against a 0.5 $\underline{mM}$ NAD^+ solution at pH 7.6 was used for the purpose of desalting in the purification procedures. The recovery during dialysis was about 90%. Desalting with a Biogel P-10 column preequilibrated with 0.5 $\underline{mM}$ NAD^+ was effective but was more complicated to perform than the short term dialysis. The effect of pH on the stability of the epimerase in the presence of 0.5 $\underline{mM}$ NAD^+ is shown in Table VII. Since the pH range where the enzyme was stable for three days was between 7.0 and 8.0, the purification involving the column chromatographic technique was performed in this range.

A heat step at 37°C, 40°C and 45°C of the alkaline ammonium sulfate preparation in the presence of 10 $\underline{mM}$ NAD^+ was tried. The epimerase lost activity rapidly by a heat treatment even in the presence of NAD^+.

The details of the purification of bovine mammary epimerase will be described. All purification steps were carried out in an ice bath at 0°C or in a cold room at 4°C unless otherwise stated.

Purification Steps

Step I: Extraction with 0.25 M Potassium Phosphate at pH 7.6

For the purification of the enzyme, 80 g of the bovine mammary
tissue acetone powder were extracted with 1,200 ml 0.25 M potassium
phosphate buffer, pH 7.6. After stirring for 60 to 70 minutes the
suspension was centrifuged at 12,000 x g for 20 minutes and the
supernatant solution was filtered through two layers of cheese cloth
to remove fatty material which floated on the top of the supernatant
solution.

Step II: 30 - 60% Ammonium Sulfate Fraction

The extract from Step I was brought to 30% saturation by adding
176 g ammonium sulfate to each liter of the extract. The percent
saturation was based on the table in reference (114) at 25°C. The
solid ammonium sulfate was added over a 30 minutes period and the
solution was stirred for 20 minutes in an ice-bath. The cloudy solu-
tion was centrifuged at 12,000 x g for 20 minutes and the precipitate
was discarded. The supernatant was brought up to 60% saturation by
adding 198 g ammonium sulfate to every liter of the 30% supernatant
solution over a period of 30 minutes. The solution was stirred for
another 20 minutes and centrifuged at 12,000 x g for 20 minutes. The
precipitate was dissolved in 25 mM potassium phosphate buffer, pH 7.6,
to a final volume of 160 ml and the supernatant solution was discarded.

Step III: Precipitation with Alkaline Ammonium Sulfate

A total of 68 ml of saturated alkaline ammonium sulfate solution
(pH adjusted to 8.0 by adding concentrated ammonium hydroxide to
saturated ammonium sulfate solution) were added slowly to 160 ml of the
enzyme solution from Step II. This cloudy solution was stirred for
15 minutes, and centrifuged at 25,000 x g for 15 minutes. The precipi-
tate was discarded. Another 52 ml of saturated alkaline ammonium
sulfate solution were added slowly to the supernatant solution. The
suspension was stirred and centrifuged. The supernatant solution was
discarded and the precipitate was dissolved in cold water to a final
volume of 40 ml.

Step IV: Negative Alumina C_r Adsorption

An aliquot of 0.1 ml of the enzyme solution obtained from Step III
was diluted 100 times to estimate the protein concentration by the
260 : 280 mμ absorbance ratio (115). The remaining enzyme solution
was dialyzed against 400 ml of 0.5 m$\underline{M}$ NAD$^+$, pH 7.6, for 2 hours. 300
ml of C_r gel (13.8 mg of solids per ml, over two years old) were added
to the dialyzed enzyme solution over a period of 15 minutes to give
a final concentration of 2 mg C_r gel per mg protein. The gel solution,
after stirring for 10 minutes, was centrifuged at 3,000 x g for 5
minutes. The precipitated C_r gel was washed with 50 ml of cold de-
ionized water and recentrifuged at 20,000 x g for 5 minutes. The
reprecipitated gel was discarded. Ammonium sulfate was added to the
combined supernatant solution until 65% saturation (430 g/l). During

the addition of the ammonium sulfate, the pH of the solution was maintained between 7.0 - 7.5 by adding 2 N ammonium hydroxide solution. The enzyme solution was centrifuged at 25,000 x g for 15 minutes. The precipitate was dissolved in 25 mM potassium phosphate buffer, pH 7.6, to a final volume of 10 ml and the supernatant solution was discarded. In case the enzyme preparation was to be frozen and stored at -20°C, the precipitate was dissolved in a minimum volume of the buffer.

Step V: Hydroxylapatite Chromatography

A column (3.5 x 12 cm) was prepared with hydroxylapatite which had been equilibrated with 0.25 M potassium phosphate buffer, pH 6.8, and washed with distilled water. The column was washed with 5 mM potassium phosphate buffer containing 0.5 mM NAD^+, pH 6.8, until the effluent solution had the same concentration of NAD^+ as the buffer as detected by the absorbance at 262 mμ. The enzyme preparation from Step IV was dialyzed against 300 ml of 0.5 mM NAD^+, pH 7.6, for two hours and was placed on the column. A stepwise gradient was used to elute the protein. Each 400 ml of 5 mM, 23 mM, 58 mM, 100 mM and 500 mM potassium phosphate buffers containing 0.5 mM NAD^+, pH 6.8, were passed through the column and fractions of 10 ml were collected at a flow rate of 1 ml per minute. In case of a slow flow rate, a LKB peristaltic pump was used to increase the flow rate. A pattern of hydroxylapatite chromatography is shown in Figure 10. Fractions containing the epimerase from the main peak were pooled and the enzyme was precipitated with ammonium sulfate at 65% saturation (430 g/l) while the pH of the solution was kept between 7.0 - 7.5 by the addition
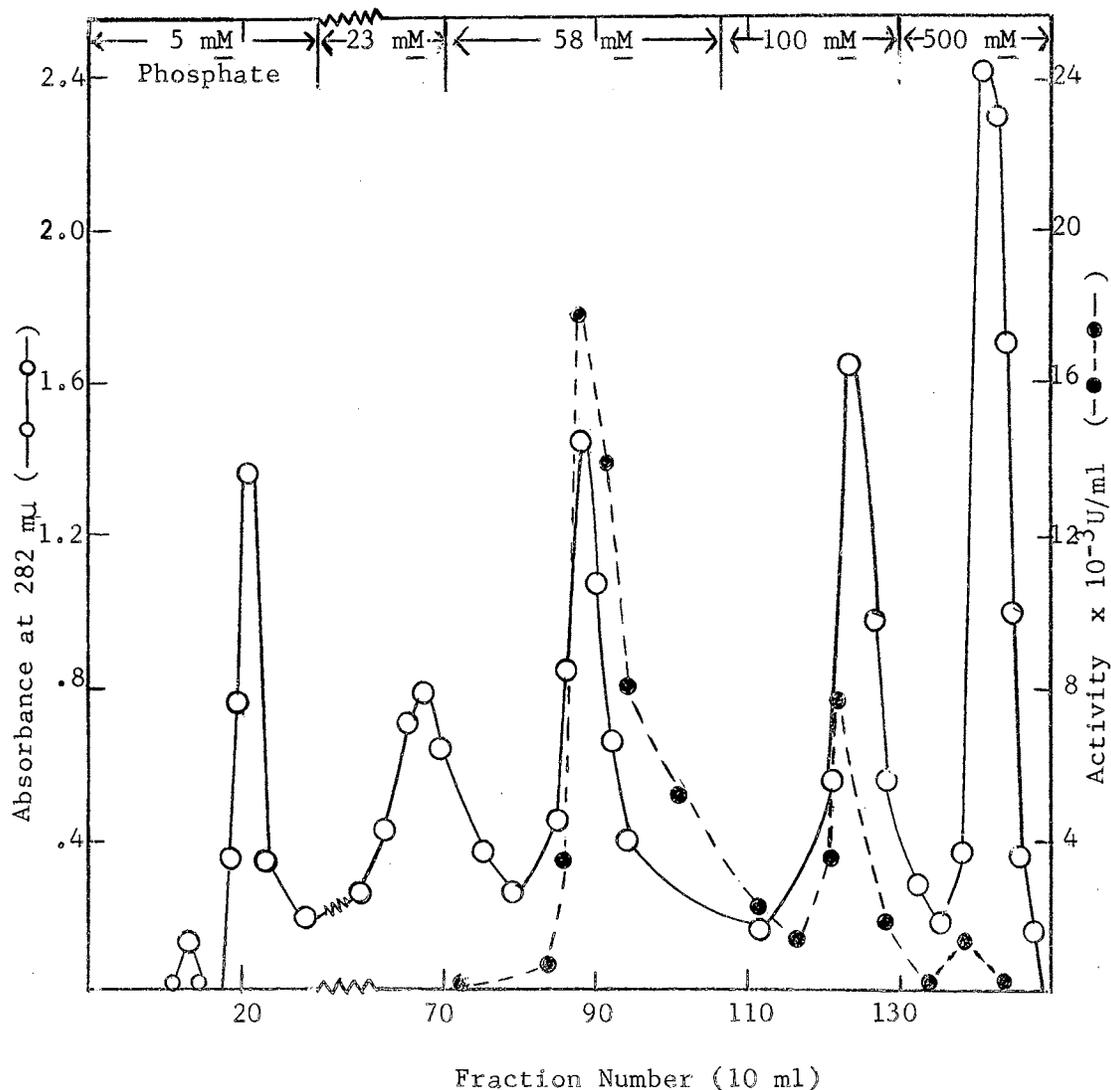
Figure 10. Hydroxylapatite Chromatography of UDP-Galactose
4-Epimerase.

The dialyzed epimerase solution from Step IV was placed
on a 3.5 x 12 cm column. A stepwise elution was used. Each
400 ml of 5, 23, 58, 100 and 500 mM potassium phosphate
buffers containing 0.5 mM NAD^+, pH 6.8, were passed through
the column. Fractions of 10 ml were collected at a flow rate
of 1 ml per minute. The concentration of protein (—O—O—)
was monitored by the absorbance at 282 mμ using buffer solu-
tion containing 0.5 mM NAD^+ as a blank. The epimerase acti-
vity (—⦿—⦿—) was determined by Assay I.

of 2 N ammonium hydroxide. The precipitate was dissolved in a small

volume (about 4 ml) of 25 mM potassium phosphate buffer, pH 7.6.

Step VI: DEAE-Cellulose Chromatography

A column (2.5 x 27 cm) was prepared with DE-32 cellulose which

had been equilibrated with 25 mM potassium phosphate buffer, pH 6.9.

The column was washed with 25 mM potassium phosphate buffer containing

0.5 mM NAD$^+$, pH 6.9, until the effluent had the same concentration of

NAD$^+$ as the washing buffer. The enzyme solution from the previous

step was dialyzed against 200 ml of 0.5 mM NAD$^+$, pH 7.6, for two hours

and then was placed on the DEAE column. A linear gradient of 25 mM

to 0.25 M potassium phosphate containing 0.5 mM NAD$^+$, pH 6.9, was

applied and the total gradient volume was 500 ml (each 250 ml). Frac-

tions of 5 ml were collected at a flow rate of 0.5 ml per minute.

The elution pattern of DEAE chromatography is shown in Figure 11.

Fractions containing the enzymatic activity were pooled and the

epimerase was precipitated at 65% saturation of ammonium sulfate while

keeping the pH of the solution between 7.0 - 7.5 by the addition of

2 M ammonium hydroxide. The precipitate was dissolved in about 1 ml

of 25 mM potassium phosphate buffer at pH 7.6. The elution pattern

on DEAE chromatography at pH 8.0 was similar to that at pH 6.9.

Step VII: Sephadex G-100 Chromatography

A 2.5 x 50 cm Sephadex G-100 column was equilibrated with 25 mM

potassium phosphate buffer containing 0.5 mM NAD$^+$, pH 7.4. The

epimerase preparation from Step VI was put on the column. The
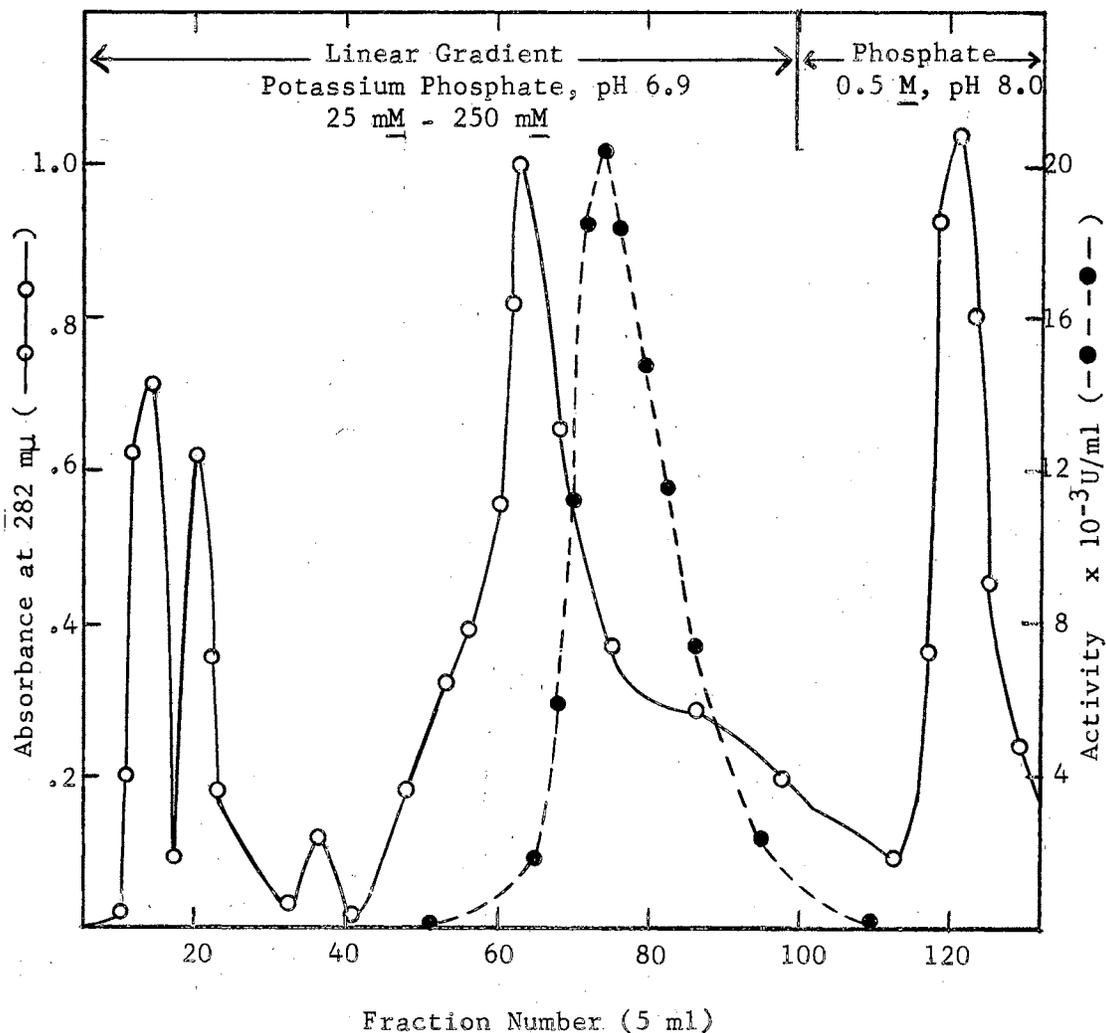
Figure 11. DEAE-Cellulose Chromatography of UDP-Galactose
4-Epimerase.

The dialyzed epimerase solution from Step V was placed
on a DEAE column (2.5 x 27 cm) equilibrated with 25 mM
potassium phosphate buffer which contained 0.5 mM NAD$^+$, pH
7.6. The epimerase was eluted with a linear gradient from
25 mM to 0.25 M potassium phosphate buffers containing 0.5
mM NAD$^+$, pH 6.9. Fractions of 5 ml were collected at a
flow rate of 0.5 ml per min. The concentration of protein
(—O——O—) was detected by the absorbance at 282 mμ using
25 mM phosphate buffer containing 0.5 mM NAD$^+$, pH 6.9 as a
blank. The epimerase activity (—●——●—) was determined
by Assay I.

epimerase was eluted with the same buffer used to equilibrate the

column at a flow rate of 18 ml per hour and fractions of 1.7 ml were

collected. The elution pattern of Sephadex G-100 chromatography is

shown in Figure 12. Fractions containing the epimerase peak were pooled

(Fractions 59 to 68). The epimerase was precipitated at 65% saturation

of ammonium sulfate while the pH of the solution was maintained between

7.0 and 7.5 by the addition of 2 N ammonium hydroxide. The precipitate

was dissolved in about 0.5 ml of 25 m$\underline{M}$ potassium phosphate buffer,

pH 7.6, and an equal volume of glycerol was mixed with it. The final

50% glycerol epimerase solution can be kept for six months at -20°C

without any significant loss of activity. The specific activity of

the epimerase purified through Step VII varied from 3 to 11 units (IUB)

per mg protein. A summary of the purification steps of bovine mammary

UDP-galactose 4-epimerase is presented in Table IX.
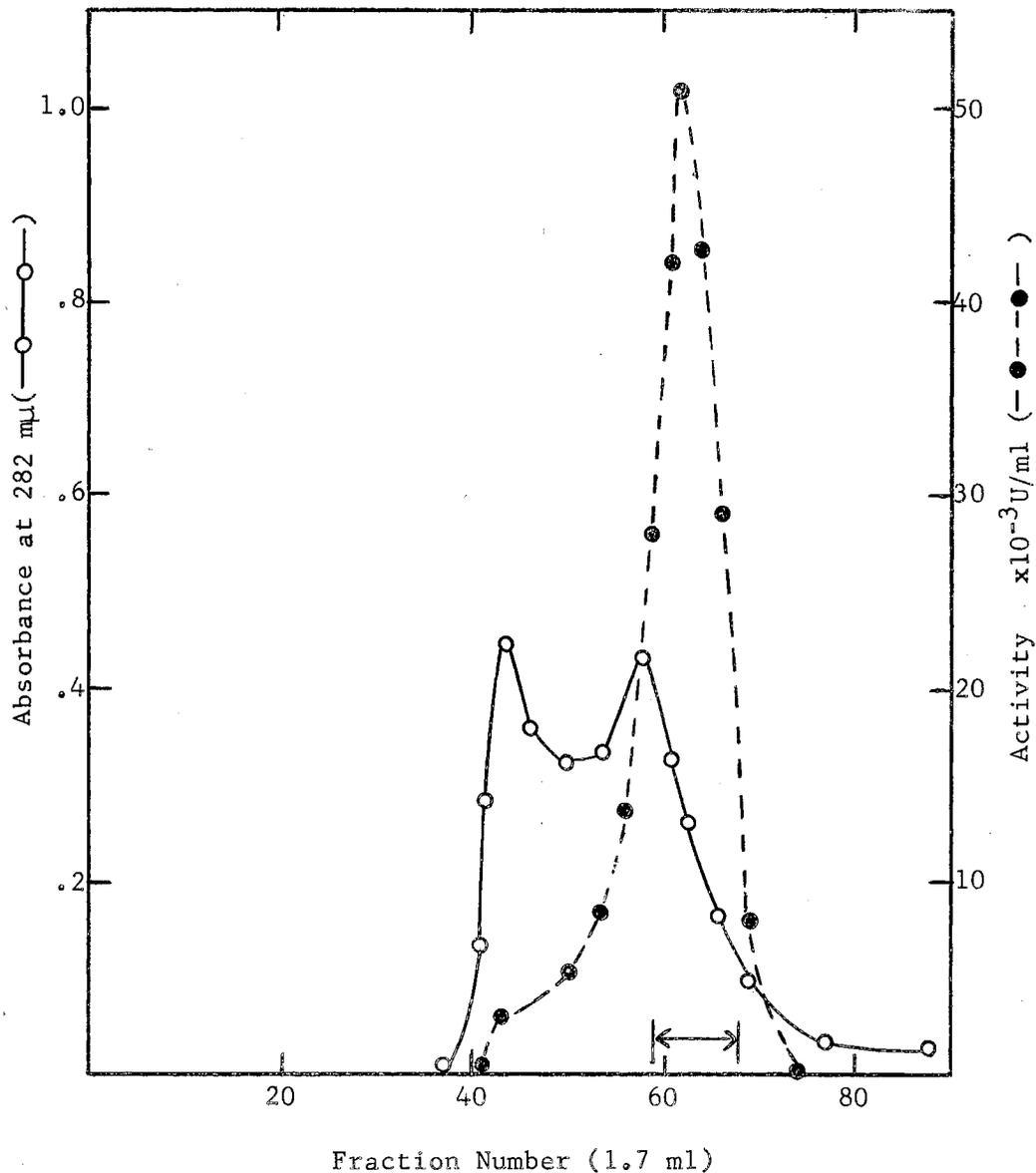
Figure 12. Sephadex G-100 Chromatography of UDP-Galactose
4-Epimerase.

The epimerase preparation from Step VI was put on a
2.5 x 50 cm column of Sephadex G-100 equilibrated with
25 mM potassium phosphate buffer which contained 0.5 mM
NAD$^+$, pH 7.4. The epimerase was eluted out with the
same buffer at a flow rate of 18 ml per hour and frac-
tions of 1.7 ml were collected. The concentration of
protein (—O——O—) was monitored by the absorbance at
282 mμ using the buffer as a blank. The epimerase acti-
vity (—●——●—) was determined by Assay I.

TABLE IX

SUMMARY OF THE PURIFICATION OF UDP-GALACTOSE 4-EPIMERASE
FROM BOVINE MAMMARY TISSUE

80 g of mammary tissue acetone powder were stirred with 1,200 ml 0.25 $\underline{M}$ potassium phosphate buffer at pH 7.6 for 65 minutes and then centrifuged at 12,000 x g for 20 minutes. The supernatant obtained was 995 ml.

		Volume	Activity	Protein	Specific Activity	Purifi- cation	Recovery
		ml	Unit(U)	mg	U/mg	Fold	%
I.	Extraction	995	221.2	11,940	.0185	1	100
II.	30 - 60% $(NH_4)_2SO_4$	160	201.6	5,856	.0344	1.9	91.2
III.	Alkaline $(NH_4)_2SO_4$	40	140.8	1,696	.083	4.5	63.6
IV.	Negative C_r Adsorption	9.2	123.6	712	.174	9.4	55.9
V.	Hydroxylapatite Column	3.8	71.4	150.5	.475	25.6	32.3
VI.	DEAE Column	1.1	40.0	18.5	2.16	116.8	18.1
VII.	Sephadex G-100 Column	17	23.5	4.9	4.76	257.0	10.6

One unit of the epimerase is defined as that amount which converts 1 μmole of UDP-galactose to 1 μmole of UDP-glucose per minute at 25°C under the conditions of Assay I.

CHAPTER VI

STABILIZATION OF UDP-GALACTOSE 4-EPIMERASE
FROM BOVINE MAMMARY TISSUES

Bovine mammary UDP-galactose 4-epimerase is labile, as shown by preliminary work done in this laboratory (66). Various efforts to stabilize the epimerase led to the use of three different kinds of reagents. These three reagents are: (1) the coenzyme NAD^+ and its analogs, (2) the substrates and their analogs, and (3) glycerol.

Stabilization of UDP-Galactose 4-Epimerase with the Coenzyme NAD^+ and Its Analogs

The epimerase is very labile when it is in a dilute solution. 25 mM potassium phosphate buffer at pH 7.6 was the best stabilizing agent (66). However, the enzyme purified through Step III lost 75% of its activity in this buffer at 4°C after one day at a protein concentration of 0.7 mg per ml. The enzyme at higher protein concentration tends to be more stable, but only 40% of its activity remained after 24 hours at 4°C at a protein concentration of 18 mg per ml.

The stability of the enzyme at 4°C was tested in the presence of 0.5 mM NAD^+ in 25 mM phosphate buffer at pH 7.6. 90% of enzymatic activity remained after one day storage in 25 mM potassium phosphate containing 0.5 mM NAD^+, pH 7.6. The stabilizing effect of NAD^+ in 25 mM potassium phosphate at pH 7.6 is shown in Table X.

TABLE X

STABILIZATION OF UDP-GALACTOSE 4-EPIMERASE
WITH NAD^+ AT 4°C

NAD^+	Activity	Units/ml					
				Days			
	0	1	2	3	7	14	28
0	1,400	340	90	---	---	---	---
0.5 mM	1,400	1,240	1,150	1,000	600	55	0

The enzyme used in this experiment was purified through Step III at a protein concentration of 0.7 mg per ml.

Since NAD^+ stabilized the epimerase, the effects of $NADP^+$ and NADH on the stability of the enzyme were tested and the results are shown in Table XI.

TABLE XI

STABILIZATION OF UDP-GALACTOSE 4-EPIMERASE
WITH $NADP^+$ AND NADH AT 4°C

	Activity	Units/ml							
					Days				
	0	1	2	3	4	5	6	7	14
0.5 mM $NADP^+$	2,150	2,100	1,670	1,620	1,520	1,710	1,570	1,300	200
0.5 mM NADH[*]	460	430	387	1,450	1,770	1,830	1,610	1,070	140

The protein concentration of the enzyme purified through Step III was 0.8 mg/ml.

[*]NADH inhibits the epimerase reaction (Table XXII).The low values in the first 2 day assays may be due to NADH inhibition of the epimerase.

The diluted enzyme solution was stabilized by 0.5 m$\underline{M}$ NAD$^+$.
A correlation between NAD$^+$ concentration and enzyme concentration on
the stabilization effect of NAD$^+$ is expected. An experiment was
carried out with various concentrations of NAD$^+$ and a partially
purified enzyme preparation at a concentration of 18 mg per ml. The
results are presented in Table XII.

TABLE XII

THE EFFECT OF NAD$^+$ CONCENTRATION ON THE STABILITY
OF UDP-GALACTOSE 4-EPIMERASE AT 4°C

NAD$^+$ Conc.	Protein Conc.	Activity x 10^{-3} units/ml						
	mg/ml			Days				
		0	1	2	3	5	7	14
0	18.0	51.3	17.8	2.4	1.4	0.6	0	---
0.5 m$\underline{M}$	18.0	49.8	42.0	4.5	2.5	0.9	0.2	---
5 m$\underline{M}$	18.0	46.5	49.5	41.0	35.0	5.0	1.0	0
10 m$\underline{M}$	18.0	47.0	46.5	53.0	43.5	32.1	16.0	2.0
20 m$\underline{M}$	18.0	52.0	46.8	45.3	40.3	34.7	17.4	3.0
20 m$\underline{M}$ at -20°C	18.0	50.0	---	---	---	---	---	40.0

The epimerase was partially purified through Step III. The
experiment was carried out at 4°C except otherwise described.

The effect of pH on the stability of the epimerase in the presence
of 0.5 m$\underline{M}$ NAD$^+$ is shown in Table VII. The pH range where the enzyme
is most stable is between 7.0 and 8.0.

TABLE XIII

STABILITY OF UDP-GALACTOSE 4-EPIMERASE IN THE PRESENCE
OF 0.5 mM NAD$^+$ AT VARIOUS pH AT 4°C

pH	Activity Units/ml						
	Days						
	0	1	2	3	5	6	8
6.0	250	160	16	---	---	---	---
6.5	305	304	254	145	47	33	---
7.0	325	332	342	330	277	225	95
7.6	353	344	352	390	252	147	46
8.0	335	330	360	312	86	52	---
8.5	333	292	210	74	47	25	---
9.0	352	256	72	39	---	---	---
9.5	317	128	35	---	---	---	---
10.0	337	50	13	---	---	---	---
10.5	309	7	---	---	---	---	---
11.0	130	---	---	---	---	---	---

The epimerase was 4 fold purified from a 0.25 mM phosphate
extract of mammary tissue acetone powder, and was diluted with
25 mM phosphate buffers at different pH values.

Stabilization of UDP-Galactose 4-Epimerase with the
Substrates UDP-Glucose and UDP-Galactose
and Their Analogs

Since NAD^+ stabilized the epimerase, it was reasonable to expect
that the substrates would also protect the enzyme. The epimerase was
stabilized at 4°C by UDP-glucose as shown in Table XIV.

TABLE XIV

STABILIZATION OF UDP-GALACTOSE 4-EPIMERASE
WITH UDP-GLUCOSE AT 4°C

UDPG Conc.	Protein Conc.	Activity x 10^{-3} units/ml							
	mg/ml	Days							
		0	1	2	5	7	10	14	30
1 mM	18.0	50.7	53.5	49.0	40.5	10.6	1.9	0.8	---
10 mM	18.0	---	31.5	34.0	34.4	33.0	33.2	23.0	0.8

The epimerase partially purified through Step III was dissolved
in 25 mM phosphate buffer containing 1 mM or 10 mM UDP-glucose at
pH 7.6.

A similar experiment with the substrate UDP-galactose was carried
out at -20°C and the results are in Table XV.

TABLE XV

STABILIZATION OF UDP-GALACTOSE 4-EPIMERASE
WITH UDP-GALACTOSE AT -20°C

UDPGal	Activity x 10^{-3} units/ml		
	Initial	2 months	5 months
0	50	29	26
1 mM	50	60	65

Both UDP-glucose and UDP-galactose stabilized the epimerase.
Therefore, one might speculate that the uracil portion of the sub-
strate binds to the enzyme and keeps the enzyme in an active conforma-
tion. A stabilization experiment with uracil, uridine and various
uridylnucleotide was carried out. The results are shown in Table XVI.

TABLE XVI

STABILITY OF UDP-GALACTOSE 4-EPIMERASE IN THE
PRESENCE OF URACIL COMPOUNDS AT 4°C

Compounds	Activity Units/ml							
	Days							
	0	1	2	3	7	10	14	28
Control (25 mM PO$_4$, pH 7.6)	1,400	340	90	---	---	---	---	---
0.5 mM UDP-Glucuronate	1,400	1,150	900	---	550	---	---	---
Saturated Uracil	1,350	240	120	---	---	---	---	---
0.5 mM Uridine	1,300	270	150	---	60	---	---	---
0.5 mM UMP	1,210	530	150	---	50	---	---	---
0.5 mM UMP-Morpholidate	1,400	530	220	---	---	---	---	---
0.5 mM UDP	1,470	1,290	1,090	1,000	770	880	300	0
0.5 mM UTP	1,000	1,150	1,080	1,000	810	1,610	920	525
0.5 mM NAD$^+$	1,440	1,240	1,150	1,000	550	110	50	0

The epimerase partially purified through Step III was diluted to
a protein concentration of 0.7 mg per ml with 25 mM phosphate buffer
containing 0.5 mM of the various nucleotides.

Results in Table X show that UDP and UTP stabilized the epimerase better than NAD$^+$. An experiment was carried out to see whether other types of nucleotides would stabilize the epimerase. The results show that ADP, ATP, CDP and GTP stabilized the epimerase to some degree but not as well as NAD$^+$ (Table XVII).

TABLE XVII

STABILITY OF UDP-GALACTOSE 4-EPIMERASE IN THE
PRESENCE OF ADP, ATP, CDP AND GTP AT 4°C

Compounds	Activity Units/ml				
	Days				
	0	1	2	5	7
Control (25 mM PO$_4$, pH 7.6)	2,000	470	130	70	40
0.5 mM NAD$^+$	2,170	1,350	1,180	650	110
0.5 mM ADP	2,100	1,280	750	150	100
0.5 mM ATP	1,970	1,110	620	170	130
0.5 mM CDP	1,900	1,100	---	200	140
0.5 mM GTP	1,950	1,300	---	350	180

Epimerase partially purified through Step III was diluted in 25 mM phosphate buffer containing various nucleotides at pH 7.6 to a concentration of 0.8 mg per ml.

Stabilization of UDP-Galactose 4-Epimerase
with Glycerol

17 β-hydroxysteroid dehydrogenase (EC 1.1.1.51) from human pla-
centa is very labile but can be stabilized by its coenzyme NAD^+, its
substrate 17 β-estradiol and also by glycerol (116). Since the labile
UDP-galactose 4-epimerase becomes stable in the presence of its co-
enzyme NAD^+ and its substrates, the stability of the epimerase was
tested in the presence of glycerol. Data in Table XII show that the
epimerase is stable in the presence of 50% glycerol at 4°C.

TABLE XVIII

EFFECT OF GLYCEROL ON STABILITY OF UDP-GALACTOSE
4-EPIMERASE AT 4°C

Glycerol	Protein Conc.	Activity x 10^{-3} units/ml					
	mg/ml			Days			
		0	1	2	3	7	14
0	30	28.0	10.0	1.8	0.9	0.1	0
20%	30	26.9	21.0	20.4	19.3	14.1	7.6
50%	30	26.8	21.5	23.6	22.5	19.1	22.8
0	1.2	1.0	0.4	0.3	0.2	0.1	---
20%	1.2	1.1	---	0.8	0.7	0.4	0.2
50%	1.2	1.1	1.1	1.0	1.1	1.1	1.2

The epimerase partially purified through Step II was dissolved
in 25 mM phosphate buffer at pH 7.6 containing various amounts of
glycerol.

The stability of the epimerase in the presence of glycerol at three different temperatures was examined and the results are shown in Table XIX. When the epimerase was stored in 20 or 50% glycerol at -20°C, it was stable for at least nine months. In 50% glycerol solution at 4°C, the epimerase was stable for one month, but was stable for only one day at room temperature.

TABLE XIX

STABILITY OF UDP-GALACTOSE 4-EPIMERASE IN THE PRESENCE
OF GLYCEROL AT DIFFERENT TEMPERATURES

Temp.	Glycerol	Activity x 10^{-3} units/ml						
		Initial	One Day	Two Days	Six Days	One Month	Six Months	Nine Months
25°C	20%	46.0	14.7	1.4	0	---	---	---
25°C	50%	45.0	40.2	23.0	4.3	---	---	---
4°C	0	54.6	26.6	9.9	1.8	---	---	---
4°C	20%	41.4	45.0	40.0	36.0	4.0	---	---
4°C	50%	48.8	45.7	41.4	40.2	41.6	6.0	---
-20°C	20%	42.8	37.8	40.5	43.0	47.0	37.3	45.0
-20°C	50%	44.0	43.5	40.0	45.0	46.0	37.5	50.0

The epimerase was partially purified through Step III. The protein concentration was 20 mg per ml in glycerol solutions containing 25 mM phosphate at pH 7.6.

It was of interest to see whether glycerol and NAD^+ have a synergistic effect on the stabilization. Data in Table XX show that 50% glycerol with 0.5 mM NAD^+ did not have any better stabilizing effect than the individual components. The 50% glycerol alone was the best agent to stabilize the epimerase.

TABLE XX

COMPARISON OF DIFFERENT STABILIZATION AGENTS AT 4°C

	Activity Units/ml							
	Days							
	0	1	2	9	17	40	70	100
25 mM PO_4, pH 7.6	1,000	420	340	70	---	---	---	---
0.5 mM NAD^+	1,050	890	1,050	120	---	---	---	---
50% Glycerol	1,130	1,110	1,000	1,100	1,220	880	430	390
50% Glycerol + 0.5 mM NAD^+	1,195	686	484	697	1,350	1,290	710	475

The epimerase was partially purified through Step III and the protein concentration was 0.7 mg per ml.

Stability of UDP-Galactose 4-Epimerase in
the Presence of Other Compounds

In order to find out whether the whole NAD^+ molecule is unique to the stabilization of the epimerase or a particular residue of the molecule also affects the stabilization, the stability of the epimerase

was tested in the presence of compounds having a part of the NAD^+

molecule. They are: niacin, nicotinamide monocleotide, adenine,

adenosine, AMP, phosphoribosyl pyrophosphate, pyrophosphate and ribose.

These compounds have no effect or very little effect on the stabiliza-

tion of the epimerase. Once the epimerase loses its catalytic acti-

vity, the activity can not be restored by incubation with NAD^+.

It was reported that the fluorescence of the yeast epimerase

increased largely in the presence of UMP and galactose (91). There-

fore, the stability of the mammary epimerase was tested in the presence

UMP and galactose, or UMP and glucose. These two combinations were

found to have no stabilizing effect.

Storage of the enzyme in suspension was also tried. A 50%

ammonium sulfate enzyme suspension was unsuccessful. The epimerase

suspension in 1.4 $\underline{M}$ K_2HPO_4 at 4°C lost about half of its activity in

a few hours, then the activity declined very slowly for a few days.

When a 1.4 $\underline{M}$ K_2HPO_4 enzyme suspension was stored at -20°C for a few

months, the activity regained its original value or even higher, and

then declined.

5 $\underline{mM}$ mercaptoethanol or cysteine, a sulfhydryl reagent, had no

stabilization effect. In some cases, it even hastened the decrease

in activity. The presence of divalent cations, such as Mg^{++}, Mm^{++}

and Zn^{++} had no effect on the stability of the enzyme. 0.25 $\underline{M}$

glycylglycine at pH 7.5 stabilized the yeast enzyme (81), but it did

not stabilize the mammary epimerase. EDTA, glucose and galactose

were also ineffective in stabilizing the epimerase.

In general, freezing and thawing resulted in a decrease in

activity of the epimerase except when the enzyme was in a 20% glycerol solution or as a 1.4 $\underline{M}$ K_2HPO_4 enzyme suspension. A 50% glycerol solution remains as a liquid even when stored at -20°C.

Deactivation and Reactivation of UDP-Galactose 4-Epimerase

In the attempts to stabilize the epimerase with NAD^+, a phenomenon of deactivation and reactivation was observed. The epimerase was partially purified through Step II and was dissolved in 250 $\underline{mM}$ potassium phosphate buffer at pH 7.4 containing 10 $\underline{mM}$ NAD^+. The protein concentration was about 20 mg per ml. The major portion of this preparation was stored at -20°C, while the rest of it was left at 4°C. After 6 days, the sample at 4°C became turbid but had more activity than before. When the turbid sample was centrifuged, all the epimerase activity was found in the supernatant portion. However, when the frozen sample was thawed after 6 days storage at -20°C, only 70% of original activity was found; when stored at 4°C, the activity dropped to 44% in 5 hours, and the activity kept declining slowly to the minimum (20% of original activity) in 4 days, then rose up to its original value in 7 days and decreased again. This deactivation-reactivation phenomenon is shown in Figure 13. The deactivation and reactivation could be hastened by a higher temperature. The epimerase reactivated to the maximum in 12 hours at room temperature, 3 - 4 hours at 37°C. Reactivation of the epimerase also occurred slowly in the 1.4 $\underline{M}$ K_2HPO_4 enzyme suspension stored at -20°C as mentioned in the previous section. This phenomenon was also observed in the dilute
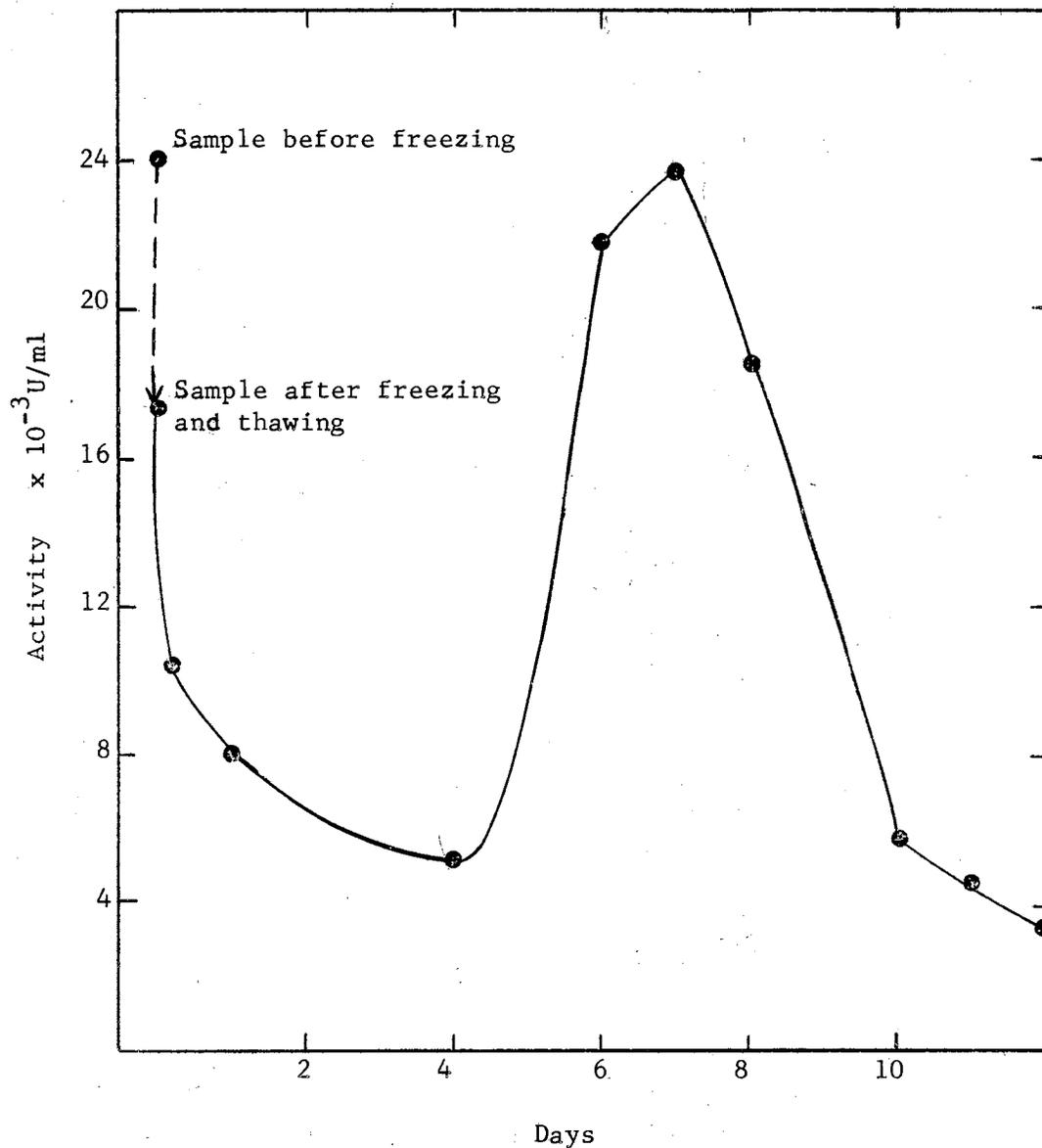
Figure 13. Deactivation and Reactivation of UDP-Galactose
4-Epimerase.

UDP-galactose 4-epimerase was partially purified through
Step II and dissolved in 0.25 M potassium phosphate at pH 7.4
containing 10 mM NAD$^+$ to give a protein concentration of about
20 mg per ml. The frozen sample was thawed after 6 days sto-
rage at -20°C and stood at 4°C during the experiment. Ali-
quots of the sample were taken out and assayed for activity
at various time.

enzyme solution in the presence of 50% glycerol and 0.5 m$\underline{M}$ NAD$^+$

(Table XIV), 0.5 m$\underline{M}$ UTP (Table X), 0.5 m$\underline{M}$ NAD$^+$ and 1 m$\underline{M}$ Zn^{++} and

sometimes in 0.5 m$\underline{M}$ NAD$^+$ though the results are variable.

CHAPTER VII

PROPERTIES OF BOVINE MAMMARY
UDP-GALACTOSE 4-EPIMERASE

Stimulation of UDP-Galactose 4-Epimerase Activity
by Exogenous NAD^+

NAD^+ is a coenzyme for UDP-galactose 4-epimerase. The purified

calf liver epimerase absolutely requires exogenous NAD^+ for activity

(79) whereas the purified yeast epimerase and the E. coli epimerase

do not require exogenous NAD^+ for activity (80, 81). Experiments were

carried out to investigate whether the purified bovine mammary

epimerase behaves like the calf liver enzyme or like the yeast and

the E. coli enzymes. A suitable amount of epimerase purified through

Step VII was incubated with 1 m$\underline{M}$ UDP-galactose in the presence of

various amounts of NAD^+ for 5 minutes. UDP-glucose formed in the

incubation mixtures was measured by Assay II. Figure 14 shows a plot

of the epimerase activity versus various concentrations of NAD^+. The

results showed that the purified bovine mammary epimerase did not

require exogenous NAD^+ for activity but did require exogenous NAD^+ to

be fully active. When the rate of UDP-glucose formed in the absence

of NAD^+ was subtracted from the rate in the presence of NAD^+ ($V - V_o$),

and a Lineweaver-Burk plot (117) of these data was made, a linear plot

was obtained. The apparent Km for NAD^+, that is, the concentration of

NAD^+ that produces half-maximal stimulation of the enzyme, was
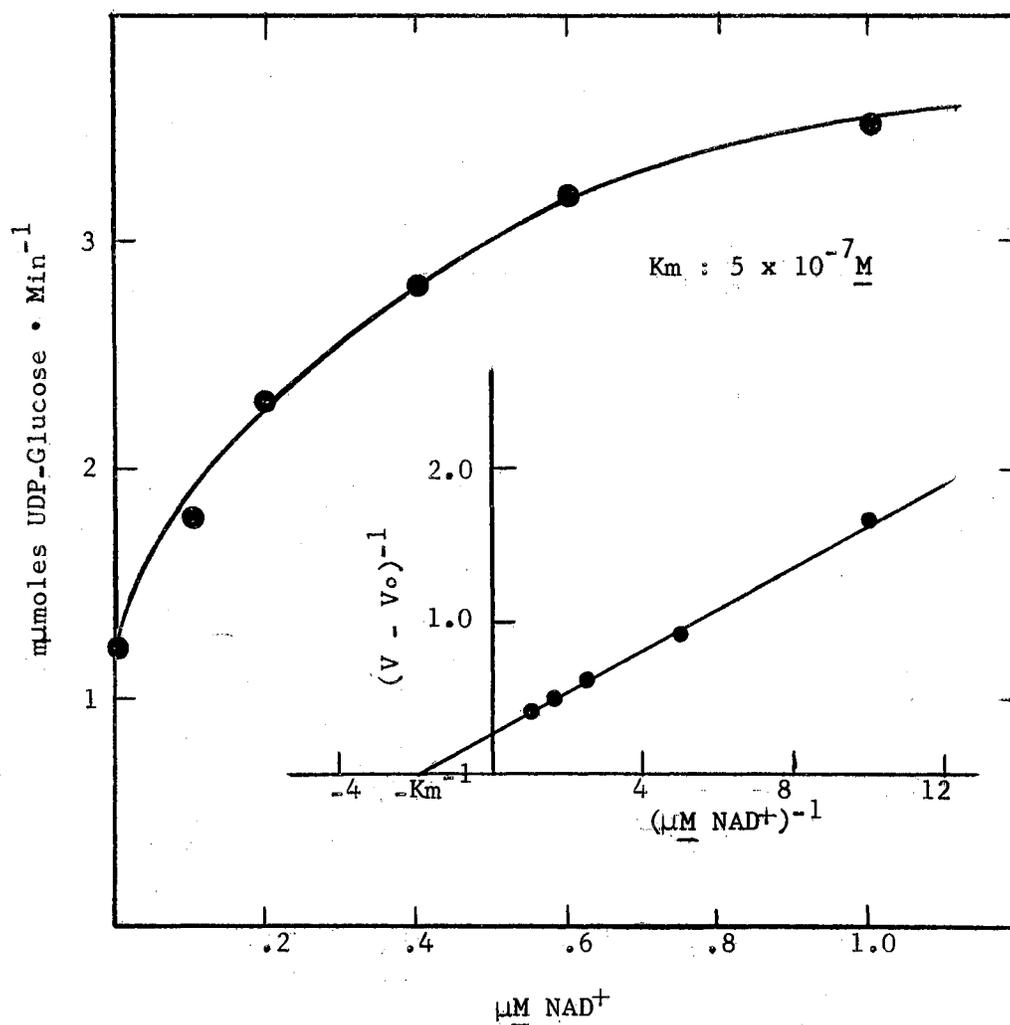
Figure 14. Rate of UDP-Glucose Formation as a Function of
NAD^+ Concentration in Assay II.

100 units of the epimerase purified through Step VII
were incubated for 5 minutes in 0.2 ml of 0.1 $\underline{M}$ glycine
buffer at pH 8.7 which had 1 m$\underline{M}$ UDP-galactose and various
concentrations of NAD^+. UDP-glucose formed in the incuba-
tion mixture was measured according to Assay II. V is
the rate in the presence of the stated concentration of
NAD^+ and V_o is the rate without NAD^+

$5 \times 10^{-7} \underline{M}.$

Since the purification of the epimerase by column chromatography was done in the presence of 0.5 m$\underline{M}$ NAD$^+$, the purified epimerase preparation might contain trace of exogenous NAD$^+$. When 9,200 units of the epimerase in 1 ml of 25 m$\underline{M}$ phosphate buffer at pH 7.6 were mixed with 4 mg of charcoal at 4°C for 3 minutes to adsorb exogenous NAD$^+$, 6,000 units of the epimerase remained in the supernatant solution after the charcoal was removed by centrifugation. Then 0.5 μmoles of UTP was added to this epimerase solution in order to stabilize the enzyme. When effect of exogenous NAD$^+$ on epimerase activity was tested on this charcoal treated epimerase, the results were similar to those obtained with the untreated enzyme. NADP$^+$ could not replace NAD$^+$ in stimulating the epimerase activity although it could stabilize the enzyme as shown in the previous chapter.

Equilibrium Constant of the UDP-Galactose 4-Epimerase Reaction

The equilibrium constant of the UDP-galactose 4-epimerase reaction was determined in both directions, from UDP-galactose towards UDP-glucose and from UDP-glucose towards UDP-galactose. A mixture of UDP-glucose and UDP-galactose can be assayed for each component by the successive use of UDP-glucose dehydrogenase and UDP-galactose 4-epimerase. The addition of NAD$^+$ and UDP-glucose dehydrogenase to the mixture results in the formation of 2 moles of NADH per mole of UDP-glucose present, and the further addition of the epimerase results in an additional 2 moles of NADH per mole of UDP-galactose present. The initial reaction mixture contains either UDP-galactose or UDP-

glucose, and UDP-galactose 4-epimerase purified through Step VII.
Aliquots were taken at various times and heated in a boiling water
bath for 3 minutes to inactive the enzyme. The results are presented
in Table XXI. The final ratio of UDP-glucose to UDP-galactose concen-
tration was 3.5 ± 0.1 whether the starting substrate was UDP-glucose
or UDP-galactose indicated that this was the equilibrium ratio.

TABLE XXI

EQUILIBRIUM CONSTANT OF UDP-GALACTOSE 4-EPIMERASE

Substrate	Incubation Time (hrs.)	UDP-glucose mμmoles	UDP-galactose mμmoles	$Keq = \dfrac{UDP\text{-}glucose}{UDP\text{-}galactose}$
UDP-Galactose	0.5	54.0	20.0	2.7
	2	59.3	18.5	3.2
	8	59.5	16.5	3.6
	24	60.5	16.5	3.6
UDP-Glucose	1	61.3	19.3	3.2
	2.5	58.4	16.9	3.5
	8	60.9	17.7	3.4
	24	60.9	18.2	3.4

Samples containing either 500 mμmoles UDP-galactose or 500
mμmoles UDP-glucose and 1,000 units of the epimerase purified through
Step VII in 0.5 ml of 0.1 M glycine buffer at pH 8.7 were incubated
at 25°C for various periods of time. Aliquots of 0.08 ml were re-
moved and boiled for 3 minutes. The amount of UDP-glucose was de-
termined first by UDP-glucose dehydrogenase as described in Assay II.
When the increase in the absorbance at 340 ceased, the amount of
UDP-galactose remaining was measured by the addition of 1,000 units
of the purified epimerase.

Km for UDP-Galactose and UDP-Glucose

The effect of UDP-galactose and UDP-glucose concentrations on the epimerase activity and the double reciprocal plots were shown in Figure 10 and Figure 16. Km values for UDP-galactose and UDP-glucose were calculated from these data according to the method of Lineweaver-Burk (117) to be $3 \times 10^{-5} \underline{M}$ for UDP-galactose and $8 \times 10^{-4} \underline{M}$ for UDP-glucose.

Inhibition Studies

Inhibition by Uridinediphosphouridine, UMP, UDP and UTP

Bovine mammary UDP-galactose 4-epimerase is inhibited by some of its substrate analogs. They are uridinediphosphouridine, UMP, UDP and UTP. The type of inhibition was determined by a double reciprocal plot with UDP-galactose as the varied substrate at several fixed level of inhibitiors, and Ki was obtained by a replot of the slope versus concentration of inhibitor (118) as shown from Figure 17 to Figure 20. Uridinediphosphouridine, UMP, UDP and UTP are all competitive inhibitors and Ki values for them are as follows: Uridinediphosphouridine, $1.8 \times 10^{-5} \underline{M}$; UMP, $5 \times 10^{-5} \underline{M}$; UDP, $3.5 \times 10^{-5} \underline{M}$; and UTP, $2.2 \times 10^{-4} \underline{M}$. Uridinediphosphouridine also inhibited the partially purified yeast epimerase. 30% and 45% of inhibition were observed at the concentrations of $1 \times 10^{-4} \underline{M}$ and $2 \times 10^{-4} \underline{M}$ respectively in Assay I.

Inhibition by NADH

NADH strongly inhibits calf liver epimerase but it does not inhibit yeast and $\underline{E}$. $\underline{coli}$ epimerases. An experiment was carried out

Figure 15.   Effect of UDP-Galactose Concentration on UDP-
Galactose 4-Epimerase Activity and Km Deter-
mination by Lineweaver-Burk Plot.

The activity of the epimerase purified through Step VII
was measured by Assay I using the stated concentration of
UDP-galactose. Activity was plotted against UDP-galactose
concentration. A Lineweaver-Burk plot was made from the
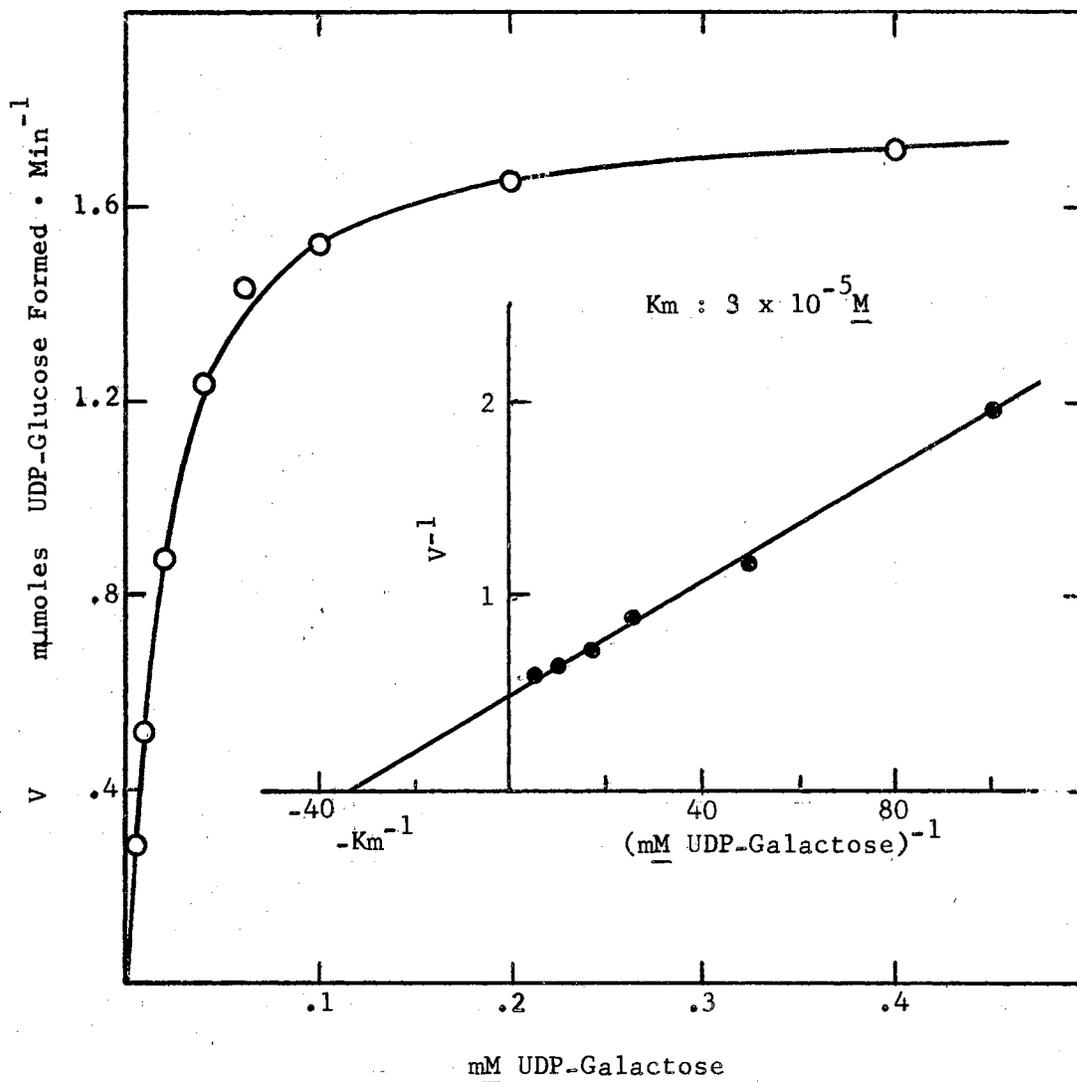data and the Km was determined by extrapolating to the base
line.
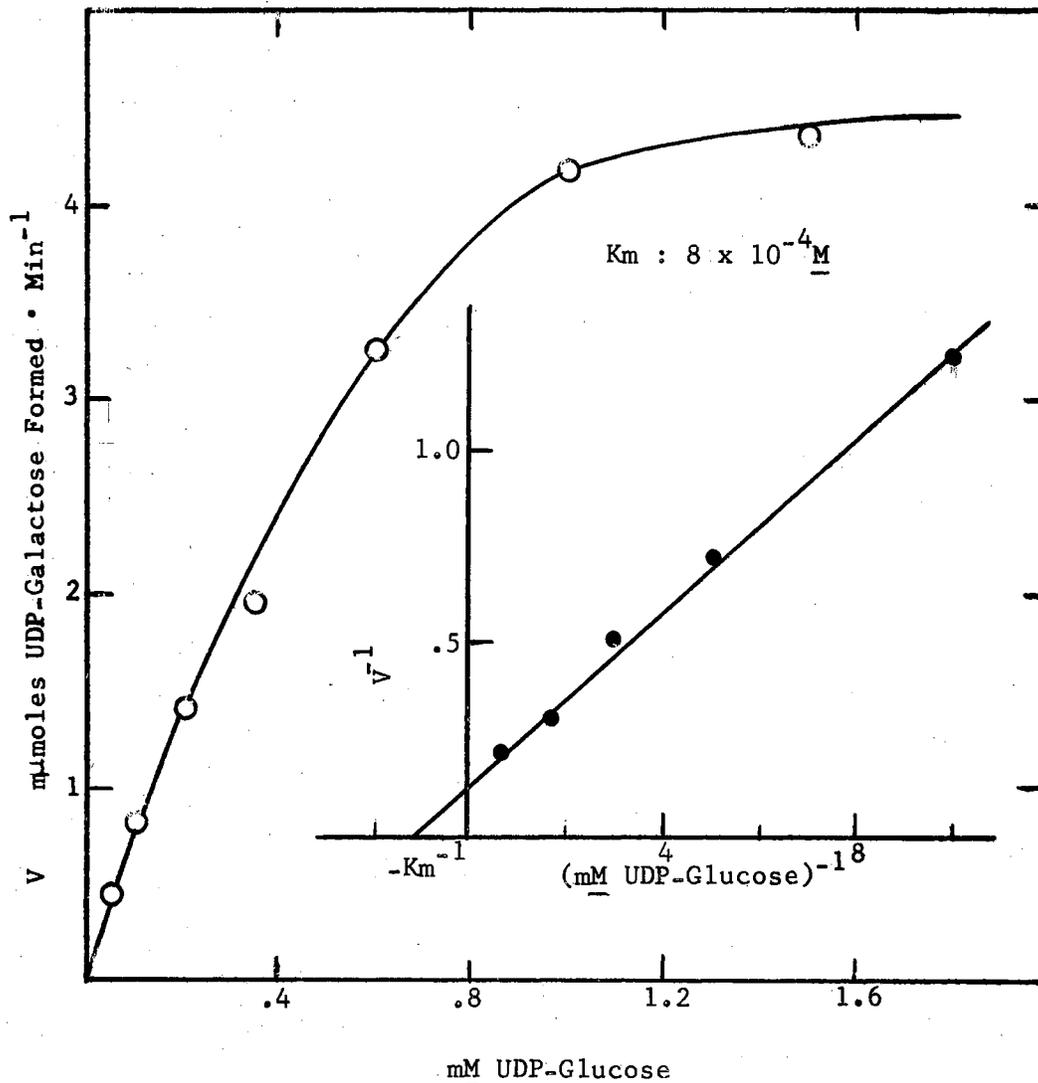
Figure 16. Effect of UDP-Glucose Concentration on UDP-
Galactose 4-Epimerase Activity and Km
Determination by Lineweaver-Burk Plot.

The activity of the epimerase purified through Step VII
was measured by Assay II using the stated concentrations of
UDP-glucose. The amount of UDP-glucose remaining after in-
cubation was calculated from the total absorbance change at
340 mµ in Assay II. The amount of UDP-galactose formed was
calculated by subtracting the amount of UDP-glucose remain-
ing from the amount in the initial reaction mixture. The
rate of UDP-galactose formation was plotted against UDP-
glucose concentration. A Lineweaver-Burk plot was made from
the data and the Km for UDP-glucose was determined.

Figure 17. Double Reciprocal Plot with UDP-Galactose as the
Varied Substrate and Uridinediphosphouridine
as the Inhibitor.

The activity of the epimerase purified through Step VII
was measured by Assay I with various concentrations of UDP-
galactose and several fixed concentrations of uridinediphos-
phouridine. The inhibitor at the highest concentration used
in the experiment did not inhibit UDP-glucose dehydrogenase.
A double reciprocal plot of the enzyme activity versus various
substrate concentrations at a fixed concentration of the in-
hibitor was made. Ki was determined by replotting the slopes
against the concentrations of the inhibitor.

Figure 18. Double Reciprocal Plot with UDP-Galactose as the Varied Substrate and UMP as the Inhibitor.

The activity of the epimerase purified through Step VII was measured by Assay I with various concentrations of UDP-galactose and several fixed concentrations of UMP. The inhibitor at the highest concentration used in the experiment did not inhibit UDP-glucose dehydrogenase. A double reciprocal plot of the enzyme activity versus various substrate concentrations at a fixed concentration of the inhibitor was made. K_i was determined by replotting the slopes against the concentrations of the inhibitor.
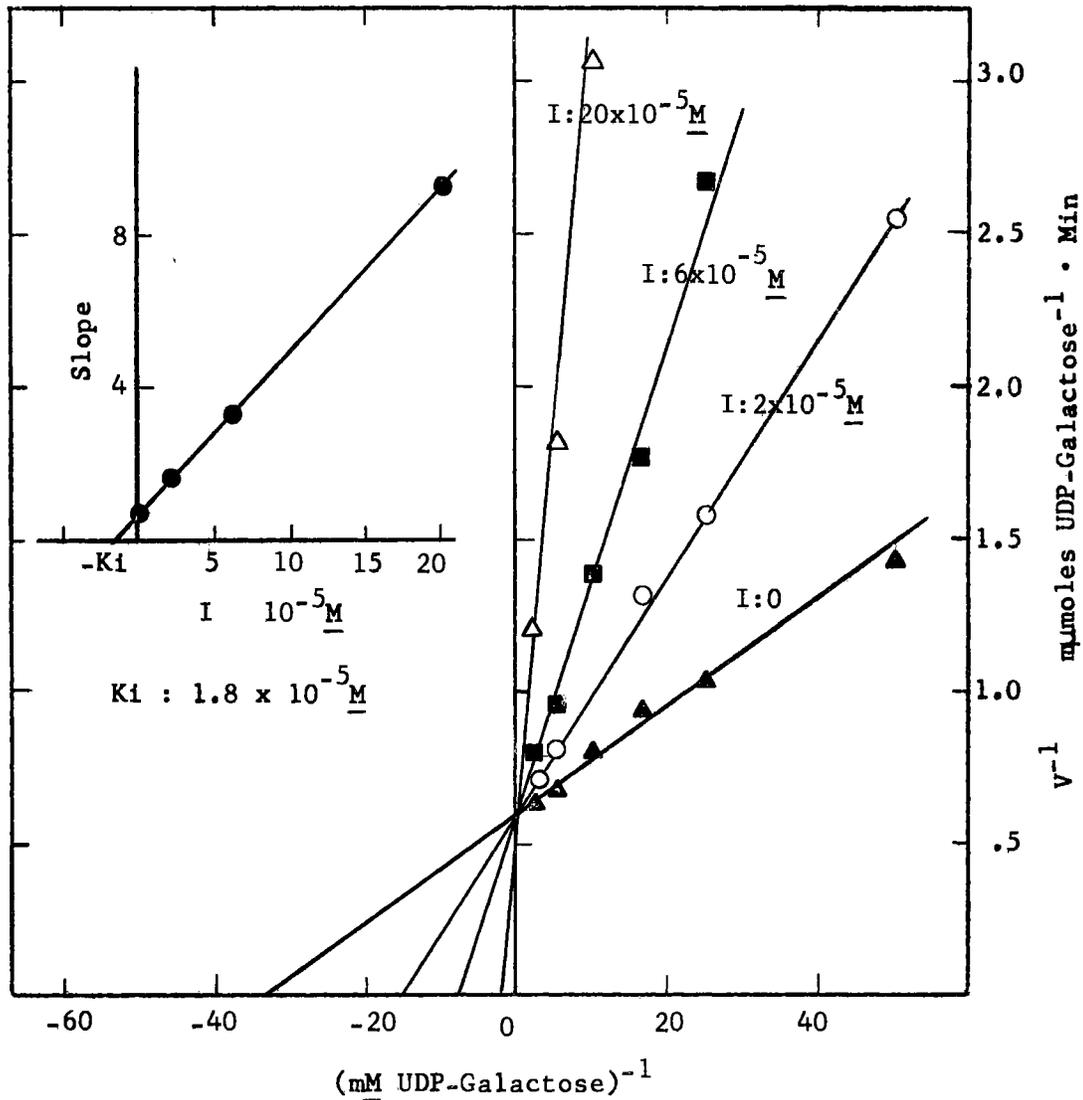
Figure 19. Double Reciprocal Plot with UDP-Galactose as the
Varied Substrate and UDP as the Inhibitor.

The activity of the epimerase purified through Step VII
was measured by Assay I with various concentrations of UDP-
galactose and several fixed concentrations of UDP. The in-
hibitor at the highest concentration used in the experiment
did not inhibit UDP-glucose dehydrogenase. A double reci-
procal plot of the enzyme activity versus various substrate
concentrations at a fixed concentration of the inhibitor was
made. Ki was determined by replotting the slopes against
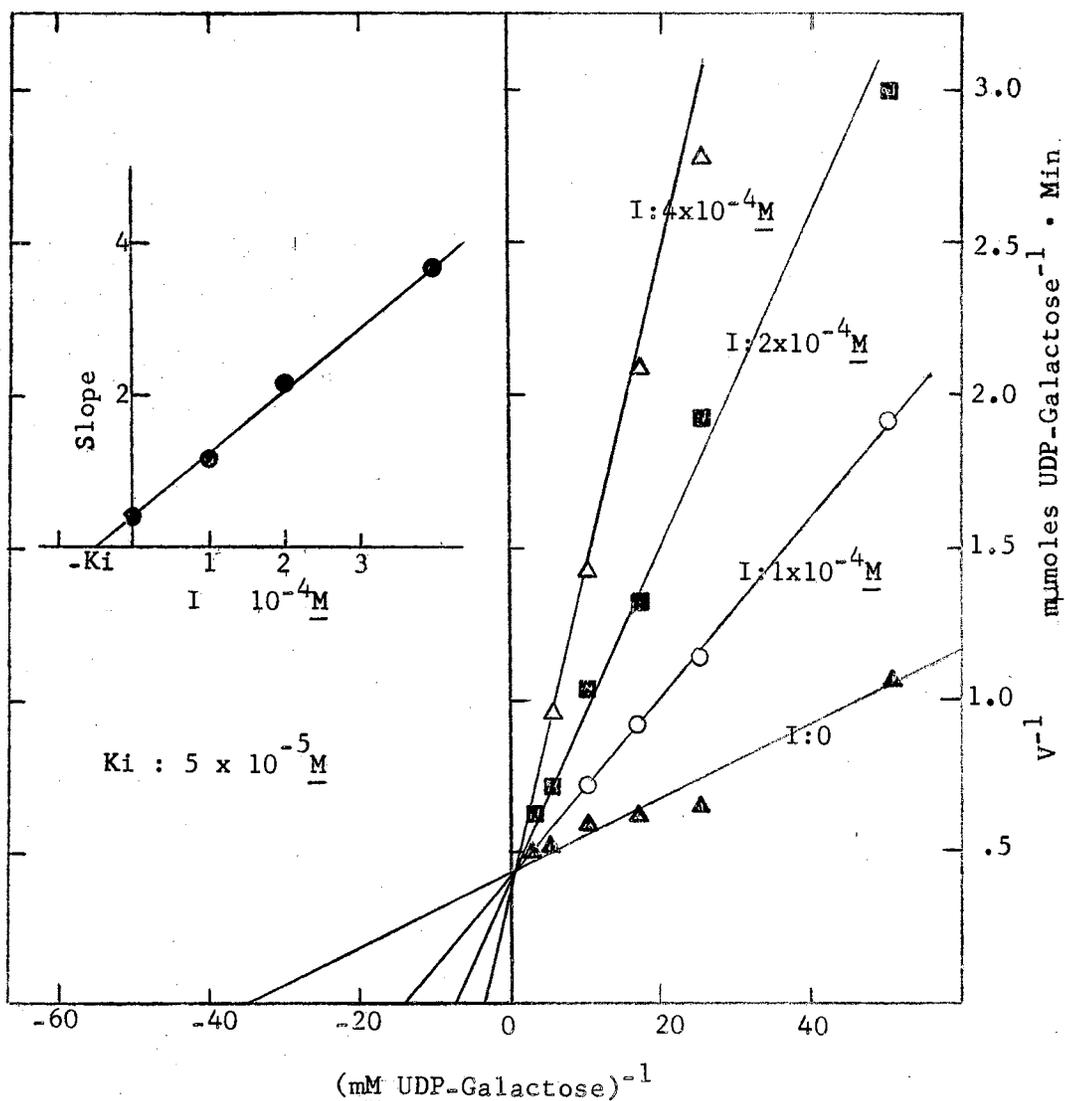the concentrations of the inhibitor.

Figure 20. Double Reciprocal Plot with UDP-Galactose as the
Varied Substrate and UTP as the Inhibitor.

The activity of the epimerase purified through Step VII
was measured by Assay I with various concentrations of UDP-
galactose and several fixed concentrations of UTP. The in-
hibitor at the highest concentration used in the experiment
did not inhibit UDP-glucose dehydrogenase. A double reci-
procal plot of the enzyme activity versus various substrate
concentrations at a fixed concentration of the inhibitor
was made. Ki was determined by replotting the slopes against
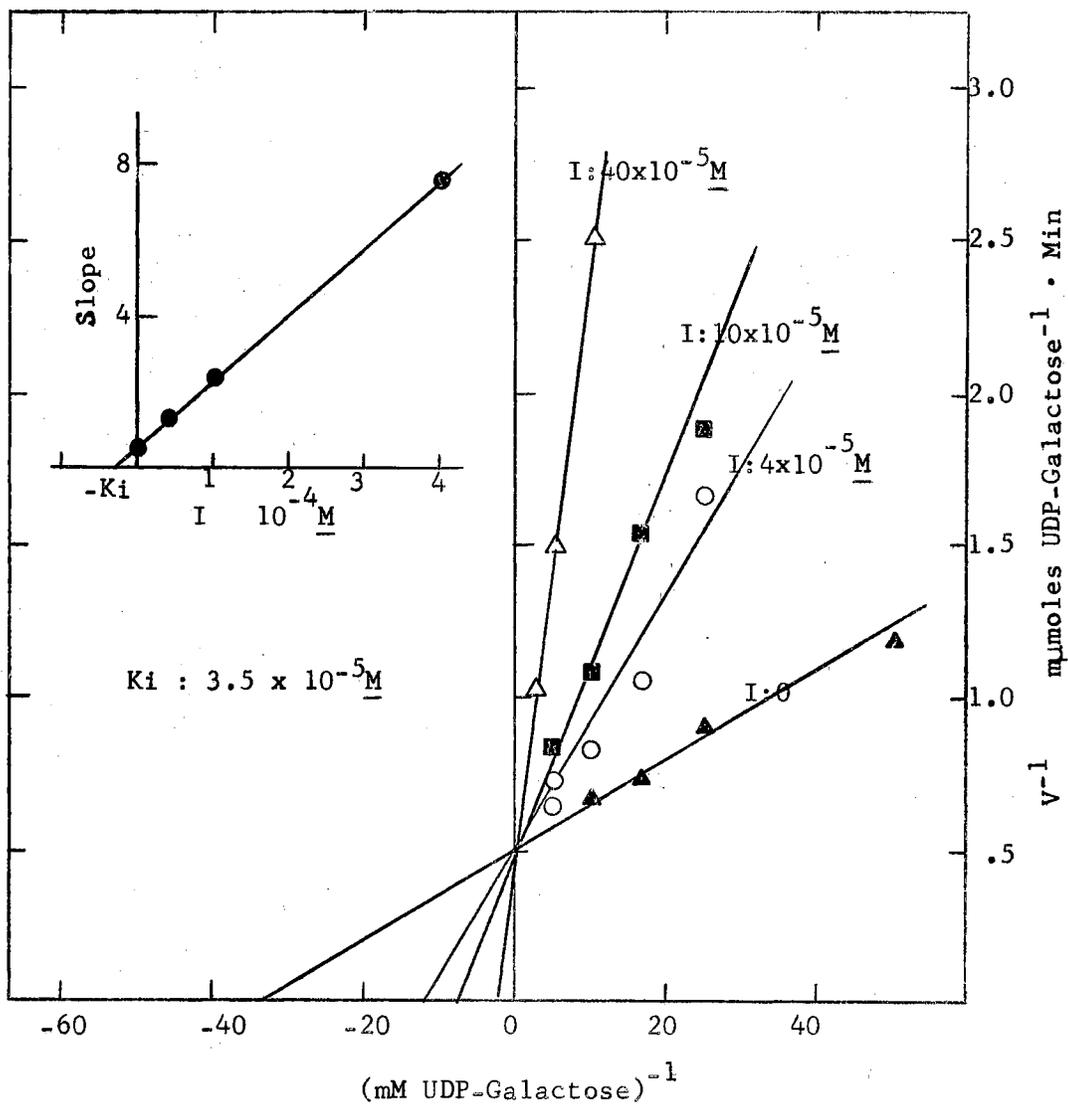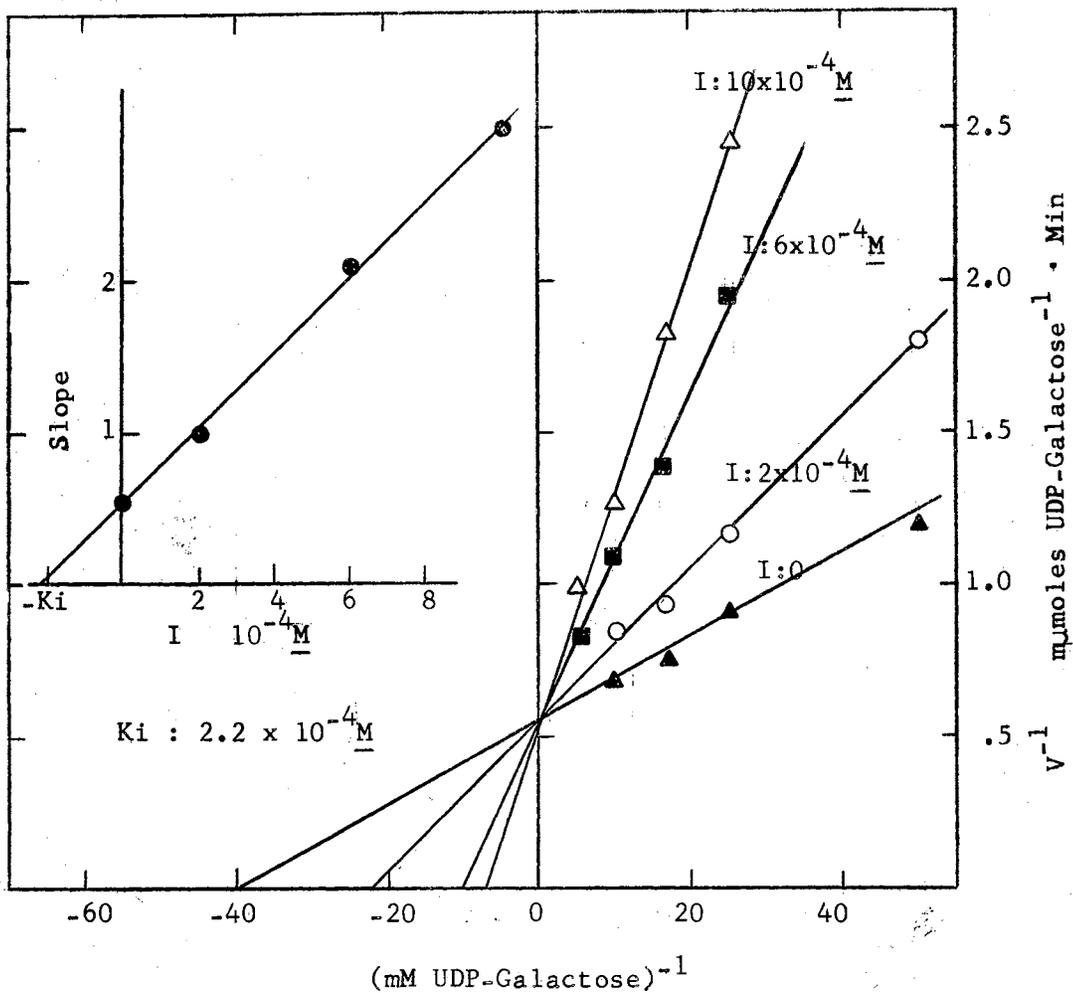the concentrations of the inhibitor.

to investigate the role of NADH in the bovine mammary epimerase.
Since NADH inhibits UDP-glucose dehydrogenase (119), the simple one-
step assay (Assay I) can not be used. Therefore, the activity of the
epimerase was measured by Assay II. Table XXII indicates that NADH
strongly inhibits bovine mammary epimerase. More than 50% of the
epimerase activity was inhibited by the presence of $5 \times 10^{-5}\underline{M}$ NADH
and $2 \times 10^{-4}\underline{M}$ NAD^+. When the concentration of NADH was increased to
that of NAD^+, 75% of the activity was inhibited. However, increasing
the concentration of NAD^+ could partially overcome the inhibition.

TABLE XXII

INHIBITION OF UDP-GALACTOSE 4-EPIMERASE BY NADH

NADH	UDP-Glucose Formed	Inhibition
$10^{-4}\underline{M}$	mμmoles	%
0	20.7	0
0.5	9.6	53.5
1	6.8	67.5
2	5.2	75.0
4	3.6	82.5
4 (+ $10^{-3}\underline{M}$ NAD^+)	6.0	71

The epimerase activity was measured by Assay II
with 95 units of the epimerase purified throught Step
VII and various concentrations of NADH as indicated.
The NAD^+ concentration was 0.2 m$\underline{M}$ except otherwise stated.

Inhibition by PCMB, Showdomycin, Iodoacetamide and Iodoacetate

The calf liver and yeast epimerases were inhibited by PCMB; however, PCMB has no effect on the $\underline{E}$, $\underline{coli}$ enzyme. Four sulfhydryl reacting compounds, PCMB, showdomycin, iodoacetamide and iodoacetate, were examined for their effect on bovine mammary epimerase. Since UDP-glucose dehydrogenase is very sensitive to sulfhydryl reacting reagents (66, 120), these compounds had to be removed by a suitable method before the epimerase activity could be assayed by coupling with UDP-glucose dehydrogenase.

The purified epimerase was incubated with showdomycin at room temperature for 10 minutes and the remaining showdomycin was reacted with excess cysteine as described by Roy-Burman $\underline{et}$ $\underline{al}$. (120). Showdomycin at a concentration of $1 \times 10^{-4}\underline{M}$ and $4 \times 10^{-4}\underline{M}$ resulted in a 9%, 20% inhibition respectively of the bovine mammary epimerase in Assay I.

The epimerase was incubated with $1 \times 10^{-4}\underline{M}$ PCMB, $5 \times 10^{-4}\underline{M}$ iodoacetamide or 5 m$\underline{M}$ iodoacetate in 25 m$\underline{M}$ phosphate buffer, pH 7.6, at 25°C for 10 minutes and the incubation mixture was passed through a 1×15 cm Biogel P-10 column, which was preequilibrated and eluted at 4°C with 25 m$\underline{M}$ potassium phosphate buffer at pH 7.6 containing 0.5 m$\underline{M}$ NAD$^+$, to separate the enzyme from the inhibitors and inorganic salt as detected by a conductivity meter. PCMB at a concentration of $1 \times 10^{-4}\underline{M}$ resulted in a 38% inhibition of the epimerase when compared with an appropriate control. Iodoacetamide $(5 \times 10^{-4}\underline{M})$ and Iodoacetate $(5 \times 10^{-3}\underline{M})$ had little inhibition on the epimerase since more than 90% of the enzymatic activity remained when compared with an appropriate control.

Determination of Molecular Weight

The molecular weight of UDP-galactose 4-epimerase was determined on the purified enzyme by use of a Sephadex G-100 column (2.5 x 50 cm) as described by Andew (119). The markers used in the experiment are blue dextran 2,000 (for determination of void volume), bovine serum albumin (67,000), β-lactoglobulin (36,500), cyctochrome C (12,400) and lactic dehydrogenase (140,000). The values in the parentheses are their molecular weights. The elution pattern of Sephadex G-100 column is shown in Figure 21. When a plot of elution volumes against molecular weight (log scale) of the marker was made, a linear relationship was obtained as shown in Figure 22. From the elution volume of the UDP-galactose 4-epimerase, the molecular weight of the enzyme was estimated to be 60,000.

Polyacrylamide Gel Disc Electrophoresis

Electrophoresis in polyacrylamide gel was performed in a Canalco disc electrophoresis apparatus Model 66 according to the manual by Canalco (Bethesda, Maryland). The standard gel (7% acrylamide) was prepared at room temperature and the electrophoresis was run at a constant current of 5 ma per gel column in a cold room at 4°C. After the indicator dye (bromphenol blue) migrated nearly the full length of the column in about 60 minutes, the gels were removed, stained overnight with amido black and destained by electrophoresis in 7.5% acetic acid at room temperature. The electrophoresis of the epimerase preparation, purified through Step VII, on polyacrylamide gel showed one
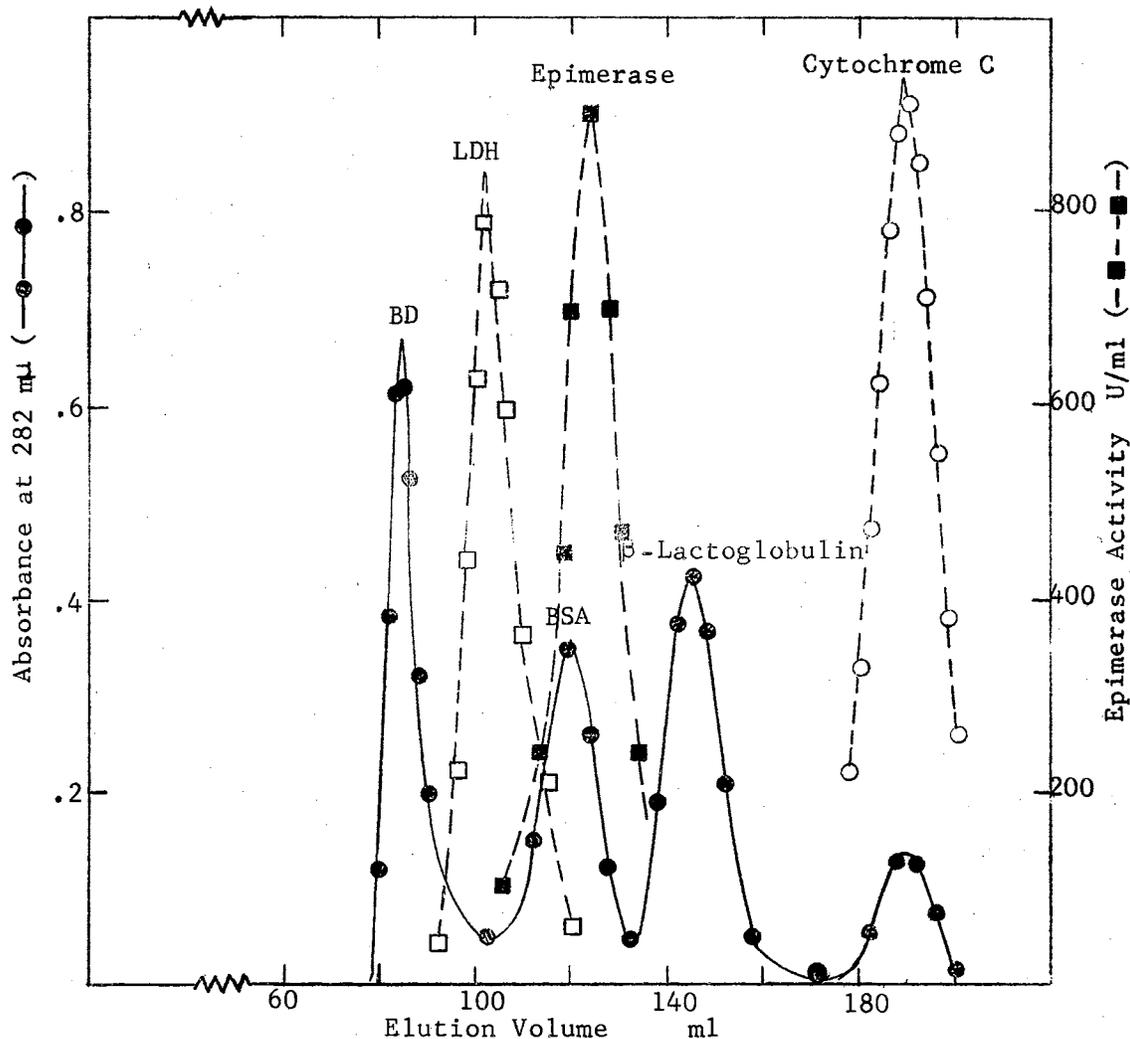
Figure 2b. Elution Pattern of Sephadex G-100 Column.

2 mg blue dextran G-2000, 8 mg bovine serum albumin, 10 mg β-lactoglobulin, 3 mg cytochrome C, 0.1 mg purified horse heart lactic dehydrogenase and 12,000 units (0.2 mg) of purified UDP-galactose 4-epimerase were dissolved in 1 ml of 25 mM phosphate buffer at pH 7.5 which contained 0.5 mM NAD$^+$ and 0.1 M KCl. The sample was loaded on a Sephadex G-100 column (2.5 x 50 cm) preequilibrated with the buffer. The column was eluted with the buffer at a flow rate of 12 ml per hour and fractions of 2 ml were collected. Blue dextran and other marker proteins were monitored by the absorbance at 282 mμ(—●——●—). Cytochrome C was rechecked by the absorbance at 412 mμ(—O——O—). The epimerase activity (—■——■—) was measured by Assay I and lactic dehydrogenase activity (—□——□—) was detected by the Assay described in Part I of this dissertation.

Figure 22. Molecular Weight of UDP-Galactose 4-Epimerase.

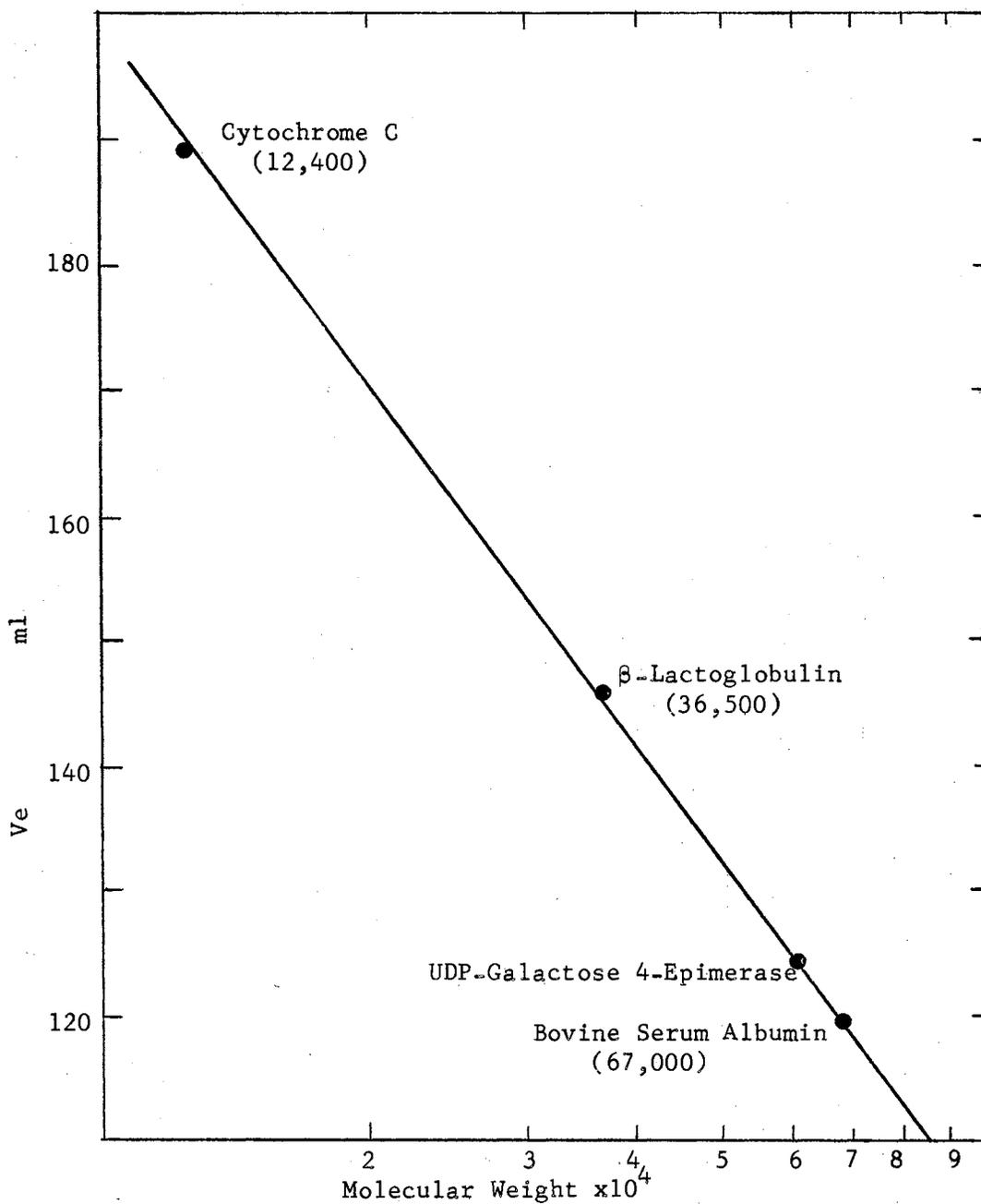A plot of elution volume (Ve) off Sephadex G-100 column against the molecular weights of bovine serum albumin (67,000), β-lactoglobulin (36,500), and cytochrome C (12,400) was made. The molecular weight of UDP-galactose 4-epimerase was determined from its elution volume to be 60,000.

sharp fast moving band and a light diffuse region on the sample end of the gel. When 0.5 m$\underline{M}$ NAD$^+$ or 0.5 m$\underline{M}$ UDP-glucose was incorporated in the polyacrylamide gel and the electrode buffer, the fast moving band appeared on the same position on the gel as before, while the light diffuse region disappeared with the formation of another sharp band located in the middle part of the gel as shown in Figure 23. The intensities of these two bands were about equal. When the fresh gel obtained from the electrophoresis in the presence of NAD$^+$ was cut into halves longitudinally and one half was stained with amido black and the other was cut into 1/8 inch sections for the epimerase assay, the epimerase activity was associated with the slower moving band.

The effect of pH on the bovine mammary UDP-galactose 4-epimerase activity was studied by Holmberg (66) in this laboratory. The pH optimum range was between 8.5 and 9.0.
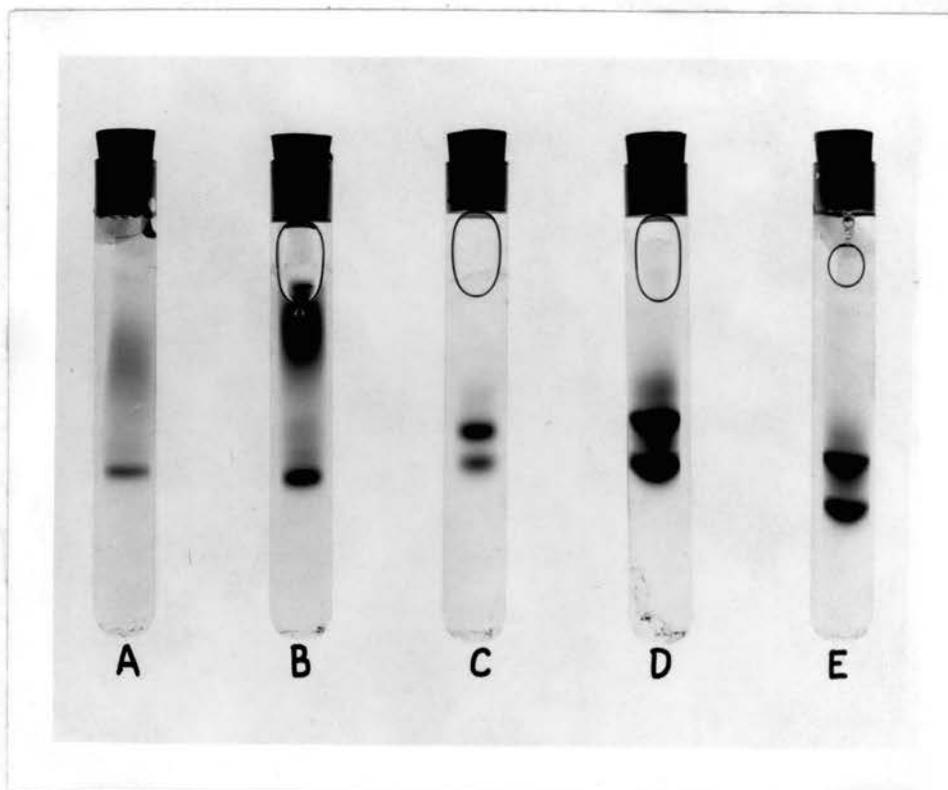
Figure 23. Electrophoretic Pattern of Purified UDP-Galactose
4-Epimerase in Polyacrylamide Gel.

     UDP-galactose 4-epimerase purified through Step VII was
subjected to electrophoresis in 7% acrylamide gel at pH 9.5
and stained with amido black. The amount of epimerase and
electrophoretic conditions are: A, 30 µg; B, 70 µg; C, 50
µg and in the presence of 0.5 mM NAD$^+$; D, 120 µg and in the
presence of 0.5 mM NAD$^+$; E, 70 µg and in the presence of
0.5 mM UDP-glucose.

CHAPTER VIII

DISCUSSION

Purification and Stabilization of Bovine Mammary
UDP-Galactose 4-Epimerase

The chief obstacle to the purification of the bovine mammary

UDP-galactose 4-eipimerase was the extreme instability of the enzyme

when it was in dilute solutions. However, when 0.5 m$\underline{M}$ NAD$^+$ was added

to the phosphate buffer, 80% of its activity remained after 3 days at

4°C as shown in Table IV. The purification of the epimerase by column

chromatographic techniques usually led to more than 50% loss of the

total activity in one day at 4°C. When a column was preequilibrated

with an appropriate buffer containing 0.5 m$\underline{M}$ NAD$^+$, the recovery of

activity off such a column was between 70 - 80%, even though the

chromatography required 3 days at 4°C.

The specific activity of the bovine mammary epimerase purified

through Step VII varied from 3 to 11 micromoles per minute per mg pro-

tein as defined by the International Union of Biochemistry. The specific

activity of purified preparations from other sources were: the calf

liver enzyme, 0.65 (79); the yeast enzyme, 40 - 90 (82); and the E. coli

enzyme, 217 (80). Although NAD$^+$ could protect the bovine mammary

epimerase, it could not completely stabilize the enzyme. More than

50% of the original activity gradually decayed during the seven purifi-

cation steps. If the mammary epimerase had been completely stabilized

and no fractionation losses occurred, the specific activity of the purified epimerase would have been three times higher.

When the purified epimerase was subjected to electrophoresis on 7% polyacrylamide gel at pH 9.5, there appeared a sharp fast moving band and a light diffuse region on the sample end. If the polyacrylamide gel was run with the incorporation of 0.5 m$\underline{M}$ NAD$^+$ or 0.5 m$\underline{M}$ UDP-glucose, the fast moving band remained on the same position on the gel while the light diffuse region disappeared with the formation of another sharp band located in the middle part of the gel as shown in Figure 18. Since the epimerase activity was only observed in the newly formed band but not in the fast moving band, the possibility that NAD$^+$ or UDP-glucose protected the epimerase during the electrophoretic process existed. In the absence of either NAD$^+$ or UDP-glucose, the epimerase might be denatured during the electrophoretic process and appeared as a light diffuse region on the sample end of a gel instead of a sharp band in the middle part of a gel. If this is true, then the purified epimerase will have only 50% purity because the intensity of these two bands which appeared on the gel in the presence of NAD$^+$ or UDP-glucose was about equal. It is also possible that a part of the epimerase was dissociated into catalytically inactive subunits which appeared as a fast moving band in the electrophoresis and the remaining epimerase was denatured, and that in the presence of NAD$^+$ or UDP-glucose, a part of the epimerase was dissociated into subunits while the rest of it was protected by NAD$^+$ or UDP-glucose.

Efforts were made to increase the specific activity of the epimerase by collecting fewer fractions from the epimerase peaks off

hydroxylapatite and DEAE-cellulose columns. However, the enzyme solution off Sephadex G-100 column became so diluted that the precipitation of the epimerase by the addition of ammonium sulfate resulted in almost complete loss of the enzymatic activity. On one occasion when 80 gm of acetone powder was used as starting material, less than 1 mg of the epimerase was recovered in a total volume of 22 ml off a Sephadex G-100. Rechromatography of an epimerase preparation off DEAE-cellulose on a DEAE-cellulose column resulted in the same poor recovery of the epimerase. In general, if the protein concentration of the epimerase solution was less than 0.1 mg per ml, the addition of ammonium sulfate to 65% saturation resulted in 70 - 90% loss of its catalytic activity. Dehydration of the diluted enzyme solution to increase protein concentration by per-evaporation, Biodryex, Polyhall or glycerol in a cold room before adding ammonium sulfate was unsuccessful since the activity decreased during the concentrating process. Adsorption of the epimerase on DEAE-cellulose and the elution of the enzyme with 0.25 $\underline{M}$ phosphate buffer at pH 7.6 was also tried but the result was unsatisfactory. Increasing the amount of starting material may solve the problem of too low enzyme concentration encountered in the Sephadex G-100 column. When the acetone powder was increased to 160 gm, the instability of the epimerase became obvious because of the prolonged purification procedures; hence the specific activity of the purified epimerase was not increased.

After the purification of the epimerase was done in the presence of 0.5 m$\underline{M}$ NAD$^+$ as the stabilizing agent, other chemicals were found to protect the enzyme. The stabilizing agents can be classified into

three catagories: (1) the coenzyme NAD^+ and its analogs, (2) the sub-strates and their analogs, and (3) glycerol. The stabilization effect of the coenzyme on the epimerase is unique to the whole molecule since various moieties of the NAD^+ molecule were unable to protect the epimerase. NADH and $NADP^+$ had the same stabilization effect as NAD^+ on the epimerase. The substrates, UDP-glucose and UDP-galactose, and their analogs, UDP and UTP were more effective than NAD^+. 50% glycerol in 25 mM phosphate at pH 7.6 could completely stabilize the epimerase at 4°C for at least two weeks as shown in Table XVIII. In 20% glycerol the bovine mammary epimerase lost its activity slowly at 4°C. However, 20% glycerol protected the enzyme as well as 0.5 mM NAD^+. The purifi-cation of the bovine mammary epimerase should be feasible in the pre-sence of 20 - 30% glycerol at 4°C without serious stability problems. 50% glycerol is an excellent storage medium for the epimerase, but it is too viscous to be used in the purification process. UDP or UTP, if used in the purification of the epimerase, is impractical because they are expensive.

Properties of Bovine Mammary UDP-Galactose 4-Epimerase Compared with the Epimerase from Other Sources

The properties of the purified bovine mammary epimerase were similar in many respects to the properties of the epimerase isolated from other sources. At equilibrium, the ratio of UDP-glucose to UDP-galactose was 3.5 : 1 for the bovine mammary epimerase. The same ratio was reported for the E. coli enzyme (80), and 3 : 1 for the yeast and calf liver enzyme (67).

The optimum pH for the bovine mammary epimerase was 8.5 to 9.0. This range was also optimum for the enzyme isolated from other sources. The K_m for UDP-galactose, 3×10^{-5} $\underline{M}$, for the bovine mammary epimerase was slightly lower than that of the enzyme from other sources. The K_m for UDP-glucose, 8×10^{-4} $\underline{M}$, was close to the value of $\underline{E. \; coli}$ epimerase.

The coenzyme NAD^+ is so tightly bound to the yeast and $\underline{E. \; coli}$ enzyme that these enzymes do not need exogenous NAD^+ for activity. However, the purified calf liver epimerase has an absolute requirement for exogenous NAD^+ for activity. Although the purified bovine mammary epimerase did not require exogenous NAD^+ for activity, yet its cata-lytic rate was increased about three fold by adding NAD^+. Since the epimerase was purified in the presence of 0.5 $m\underline{M}$ NAD^+, it was likely that the purified enzyme contained traces of free NAD^+. However, when the epimerase was first treated with charcoal to remove free NAD^+, the same extent of stimulation was observed when NAD^+ was added. These results would suggest that NAD^+ was fairly tightly bound to the bovine mammary epimerase. NAD^+ is probably loosely bound to the calf liver enzyme and removed completely during an extensive purification. It is possible that when the bovine mammary epimerase is purified in the presence of glycerol instead of 0.5 $m\underline{M}$ NAD^+, the enzyme has an absolute requirement for exogenous NAD^+. The bovine mammary epimerase was also similar to the calf liver enzyme in that NAD^+ could not be replaced by $NADP^+$ and NADH strongly inhibited the epimerase reaction.

PCMB inhibited the calf liver epimerase and the yeast epimerase but not the $\underline{E. \; coli}$ enzyme. The bovine mammary epimerase was inhibited

by PCMB and showdomycin but was not sensitive to iodoacetate and iodoacetamide.

To determine the homogeneity and the molecular weight of the enzyme, an ultracentrifugation was run with the purified epimerase. The Schlieren pattern boundary moved too slowly due to the viscosity of the sample, which contained 15% glycerol, and the prolonged centrifugation (4 hours) flattened the boundary, and thus the attempt was unsuccessful. The molecular weight of the bovine mammary epimerase was 60,000 when determined by a Sephadex G-100 column. The E. coli enzyme has a molecular weight of 79,000 (80) and the yeast enzyme is 125,000 (82), about twice the bovine mammary enzyme.

Deactivation and then reactivation of the bovine mammary epimerase in the presence of NAD^+ was observed as shown in Figure 13. A similar phenomenon was described by Kalckar et al. (92) with yeast epimerase. The increase in the activity of UDP-galactose 4-epimerase and UDP-glucose pyrophosphorylase in the extract of the mammary tissue from various species has been investigated in this laboratory (122). The deactivation and reactivation phenomenon is still a puzzle. However, it is speculated that in the presence of NAD^+, the bovine mammary epimerase may undergo a slow temperature dependent conformational change. Perhaps this is a change from the α-helical structure to the β-structure as occurred in the yeast epimerase (92). During this conformational change, the enzyme bound NAD^+ may be gradually reduced to NADH which has a strong inhibition effect on the enzyme. Thus, the reduction of NAD^+ to NADH results in a decrease in the activity and an increase in fluorescence. When the NADH content of the epimerase

reaches a maximum, it will correspond to a maximum in fluorescence and a minimum in activity. Then the content of NADH which decreases for some reason during the conformational change may account for the reactivation of the epimerase. The eventual change from α-helical structure to β-structure causes the denaturation of the enzyme. This postulation stems from the observation that the addition of UMP and galactose to the yeast epimerase caused a conformational change and there is an inverse relationship between the fluorescence (reflection of NADH) and the activity (92).

The phenomenon of deactivation and reactivation of the epimerase may also be explained by the dissociation of the active enzyme into inactive subunits, a conformational change in the subunits, and subsequent reassociation of subunits to a new form of active enzyme which is more labile than the native enzyme. Suppose the dissociation and reassociation is in a dynamic state, the deactivation will happen in a stage where the dissociation is predominant and reactivation is in a state where reassociation is predominant. Since the new form of enzyme is supposed to be more labile, it will be denatured eventually and catalytic activity will disappear.

In the yeast epimerase, UMP caused a large increase in fluorescence in the presence of specific sugars, i.e., D-glucose, D-galactose, D-fucose and L-arabinose. However, the substrates, UDP-galactose and UDP-glucose, caused little increase in fluorescence under the same conditions. Based on this observation, Kalckar et al. (92) proposed that there are different binding sites for UMP and the substrates. In bovine mammary epimerase, UMP is a competitive inhibitor for the

substrates as shown in Figure 13. This suggests that the bovine mammary epimerase has the same binding site for UMP and the substrates, or has two binding sites so close to each other that the binding of UMP and substrates are mutually exclusive.

SUMMARY

Bovine mammary UDP-galactose 4-epimerase was purified 260 fold from an extract of mammary tissue acetone powder in 10% yield. The enzyme was labile and was stabilized by (1) the coenzyme NAD^+ and its analogs, NADH and $NADP^+$, (2) the substrates UDP-galactose and UDP-glucose and their analogs, UDP-glucuronate, UDP and UTP, and (3) 20 - 50% glycerol. Nucleotide di and tri-phosphates, such as ADP, ATP, CDP and GTP, also had some protective effect. The Km for UDP-galactose and UDP-glucose were $3.0 \times 10^{-5}\underline{M}$ and $8.0 \times 10^{-4}\underline{M}$ respectively. At equilibrium, the ratio of UDP-glucose to UDP-galactose was 3.5 : 1. The optimum pH range of the enzyme was 8.5 to 9.0. The epimerase did not require exogenous NAD^+ for its activity, but its catalytic activity could be increased 3 fold by the addition of NAD^+. The apparent Km for NAD^+ was $5 \times 10^{-7}\underline{M}$. UMP, UDP, UTP and uridinediphosphouridine were competitive inhibitors of the substrates and their Kis' were $5 \times 10^{-5}\underline{M}$, $3.5 \times 10^{-5}\underline{M}$, $2.2 \times 10^{-4}\underline{M}$ and $1.8 \times 10^{-5}\underline{M}$ respectively. The enzyme was strongly inhibited by NADH. Equal concentrations ($2 \times 10^{-4}\underline{M}$) of NAD^+ and NADH gave 75% inhibition. PCMB at $1 \times 10^{-4}\underline{M}$ caused 38% inhibition; showdomycin at $4 \times 10^{-4}\underline{M}$ caused 20% inhibition; iodoacetate at 5×10^{-3} $\underline{M}$ caused 10% inhibition. The molecular weight of bovine mammary epimerase was approximately 60,000 as estimated by a Sephadex G-100 column.

A total of 2 gm of UDP-galactose was synthesized by the method

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of Roseman et al. (106). The chemical synthesis and characterization
of uridinediphosphouridine, one of the inhibitors of the epimerase,
were described.

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