MANNOSE-6-PHOSPHATE CONCENTRATIONS IN HEPATOCYTES AND PERFUSED RAT LIVERS

By

SUDARAT MANOCHIOPINIG

Bachelor of Science in Medical Technology
Mahidol University
Bangkok, Thailand
1972

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1977

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
Doctor of Philosophy
December, 1981

MANNOSE-6-PHOSPHATE CONCENTRATIONS

IN HEPATOCYTES AND PERFUSED

RAT LIVERS

Thesis Approved:

Roger E. Knopke
Thesis Adviser

Robert K. Gholson

Ulrich K. Melcher

E. J. Eisenbraun

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author extends special appreciation to her major professor, Dr. R. E. Koeppe, for his guidance, advice and understanding. Appreciation is also expressed to Dr. R. K. Gholson, Dr. U. K. Melcher and Dr. E. J. Eisenbraun for their help as advisory committee members.

The author thanks Dr. J. A. Ontko at the Oklahoma Medical Research Foundation for his guidance and advice on the isolation of hepatocytes.

Special thanks are offered to Dr. H. O. Spivey and Dr. J. M. Merz for their guidance and assistance during this study; without their guidance this work would never have been done.

The author is indebted to Royal Thai Governments and Biochemistry Department of Oklahoma State University for the financial support that has made this work possible. Grateful acknowledgment is also extended to my friends, Sompis Hongham and Chitti Suwanwela for their valuable assistance.

Deep gratitude is expressed to my parents, Mr. Yeo Kiang Lo and Mrs. Sin Eng Erng, for their love, understanding and encouragement. A special note of appreciation is extended to my brothers and sister for their love and encouragement across the sea.

Sincere thanks are extended to Mrs. Sue Heil for typing this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

ATP	-	Adenosine triphosphate
°C	-	Degrees centigrade
EDTA	-	Ethylenedinitrolotetraacetate
EtOH	-	Ethanol
Fru-6-P	-	Fructose-6-phosphate
g	-	Gram
G-6-PDH	-	Glucose-6-phosphate dehydrogenase
Glc-1-P	-	Glucose-1-phosphate
Glc-6-P	-	Glucose-6-phosphate
Glc-1,6-diP	-	Glucose-1,6-diphosphate
GDP-Man	-	Guanosine diphosphomannose
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	-	Hexokinase
hr	-	Hour
Km	-	Michaelis constant
M	-	Molar
Man-1-P	-	Mannose-1-phosphate
Man-6-P	-	Mannose-6-phosphate
min	-	Minute
mM	-	Millimolar
NADP(H)	-	Nicotinamide adeninedinucleotide phosphate (reduced)
6-PGlc	-	6-Phosphogluconate
PCA	-	Perchloric acid

PGI - Phosphoglucose isomerase

PGM - Phosphoglucomutase

PMI - Phosphomannose isomerase

PMM - Phosphomannomutase

RNA - Ribonucleic acid

rpm - Revolutions per minute

TEA - Triethanolamine

TES - N-tris-(hydroxymethyl)methyl-2-amino-ethanesulfonic acid

Tricine - N-tris-(hydroxymethyl)methylglycine

µl - Microliter

µm - Micrometer

µM - Micromolar

Vmax - Maximum Velocity

wt - Weight

CHAPTER I

INTRODUCTION

The fact that liver occupies a central position in body metabolism, coupled with its size, softness, and relative homogeneity, have made it a favorite organ for biochemical investigation. Liver provides a constant source of glucose to meet energy requirements of peripheral tissue, removes blood lactate, is a major site of glycogen and fat soluble vitamin storage, regulates the concentration of most plasma constituents, removes metabolic end products such as bilirubin and other hemochromogens resulting from red cell destruction, and detoxifies a variety of exogenous poisons. It also regulates amino acid metabolism, synthesizes urea and plasma proteins, and regulates fat transport. Clearly, the liver's metabolic function must be viewed in relation to the functional activity and demands of peripheral tissue. Measurements of physiologic and metabolic responses under a variety of conditions are difficult because of the anatomic inaccessibility of hepatic vascular connections with the rest of the body and the subtle nature of hepatic functions.

Liver function may be investigated by using a spectrum of preparations ranging from isolated liver, subcellular organelles, tissue culture cells, liver slices, the perfused organ and the hepatectomized animal to the intact animal.

Perfusion of Isolated Liver

Perfusion of isolated liver has been widely used to investigate hepatic physiologic and metabolic reactions. The perfusion technique has several advantages over other methods for investigating liver physiology. Perfusion allows greater control of the concentration of perfusate constituents, of temperature and of other physiological conditions than is possible with intact animal preparations. It was reported that perfused rat liver performs most liver functions as does the whole animal (1, 2).

The first to use perfused rat liver were Corey and Britton in 1941 (3), Trowell in 1942 (4), Brauer, Passotti and Pizzolato in 1951 (5) and Miller, Bly, Watson and Bale in 1951 (6). Hems, Ross, Berry and Krebs (7) modified the perfusion techniques of Miller (6) and Schimassek (8) to one suitable for studying carbohydrate metabolism.

The nutritional state of an animal plays an important role in body metabolism. Fed versus fasted animals has been the choice in many metabolic studies. For carbohydrate metabolism, it is well known that fasted liver tends to synthesize glucose from gluconeogenic precursors in order to maintain blood sugar levels.

The carbohydrate metabolism of perfused rat liver has been investigated by Hems et al. (7) and Ross et al. (9, 10). They reported that the gluconeogenic capacity of livers in the well fed state is lower than that of livers under conditions where the supply of glucose is decreased, such as in starvation. The perfused liver appears to regulate the deposition of glycogen like the intact animal does. They (7, 9, 10) observed that a low concentration of glucose in the perfusing

medium causes liver glycogen degradation whereas a high concentration of glucose in the perfused medium causes glycogen deposition.

Soboll et al. (11) reported that perfused liver from fed rats has a high rate of carbohydrate breakdown and a low rate of fatty acid oxidation compared to liver from fasted rats which has increased fatty acid oxidation and gluconeogenesis from endogenous sources, rather than from glycogenolysis. It is the cytosolic proteins that are degraded during fasting to provide substrate of gluconeogenesis.

Perfused liver from fed versus fasted rats was also studied by Brunengraber et al. (12), who reported that the rate of fatty acid synthesis from a high glucose perfusate concentration (25 mM) or a low glucose concentration (4 mM) in liver from fed rats (at a rate of 170 μmole and 72 μmole of acetyl group incorporated into fatty acids per g dry weight per hour for high and low glucose concentrations, respectively) was higher than that in liver from fasted rats (at a rate of 73 μmole and 25 μmole of acetyl group incorporated into fatty acids per g dry weight per hour for high and low glucose concentrations, respectively). The relation between the glucose concentration in the perfusate and the rate of fatty acid synthesis is probably due to the high Km of glucokinase (see page 8). Furthermore, they reported that concentrations of Glc-6-P and Fru-6-P in perfused liver from fed rats were higher than those from fasted rats. The values are as follows: fed liver perfused with either 25 mM or 4 mM glucose has Glc-6-P 0.56, Fru-6-P 0.17; and Glc-6-P 0.16, Fru-6-P 0.063 μmole/g dry weight, respectively; one day or two day fasted liver perfused with 25 mM or 4 mM glucose has Glc-6-P 0.11, Fru-6-P 0.048, and Glc-6-P 0.079, Fru-6-P 0.031 μmole/g dry weight, respectively.

Although the isolated perfused rat liver has been widely used, there are several major problems such as 1) liver is not a completely homogeneous organ, containing up to 40% of non-parenchymal cells (13), 2) it is difficult to obtain many identical samples from one liver, 3) the viability of an isolated liver can be maintained for only 8-10 hr.

Methods of Isolation of Liver Cells

Isolated hepatocytes are a very valuable system for studying the interrelationships and control of liver metabolic processes. A homogeneous suspension of hepatocytes, in contrast to perfused liver, may be sampled repeatedly for measurements during the course of an experiment. Over the past twenty years preparations of isolated liver cells have been used for the study of hepatic metabolism. A variety of methods have been devised for the preparation of isolated parenchymal cells from liver. These fit into two main categories: mechanical and enzymatic.

1. Mechanical methods for parenchymal liver cell separation. Mechanical force has to be employed to disrupt the organ and liberate the individual liver cells from connective and vascular tissue. Without pretreatment, these methods require drastic mechanical procedures including homogenization (14, 15), forcing tissue through screens of stainless steel, silk, or cheese-cloth (16, 17), pipetting (18), shaking with glassbeads (19) and perfusion of liver with Ca^{+2} chelators (20).

Anderson (21) reported the first method of preparation of isolated liver cells from rats. Livers were perfused with an isotonic Ca^{+2} free

solution containing a calcium chelating agent, EDTA. Then the liver was cut into small pieces, homogenized, spun and resuspended many times. However, other investigators have been unable to obtain the high yields of cells reported by Anderson.

Schreiber (22) used a combination of Ca^{+2} chelator (citrate or EDTA) and mechanical treatment. The reason for Ca^{+2} removal, by washing or chelation, is that it has a role in cellular adhesion (23, 24).

Although cells prepared by techniques mentioned above are almost always damaged, they are useful for some studies; e.g. mitochondrial respiration apparently does not require complete cellular integrity.

2. Enzymatic techniques for parenchymal liver cell separation. In order to disperse liver slices into single cells several enzymes, alone, or in combination, have been used. Trypsin, a proteolytic enzyme, disperses liver but also destroys the parenchymal cells (19, 25-27); lysozyme (28, 29) has been used, but Seglen (30) found it completely ineffective. Many investigators tried without success, to separate liver cells before collagenase became commercially available. Collagenase was first isolated and characterized from Clostridium histolyticum by Mandl et al. (31). In 1967, great progress was made when Howard et al. (32) introduced the use of collagenase as a liver dispersing enzyme. His method was the first capable of producing a significant number of intact liver cells. By using a combination of collagenase and hyaluronidase for dispersion of liver slices, a suspension of intact liver cells was obtained. Two years later, when Berry and Friend (33) introduced the use of physiological liver perfusion (at $37^{o}C$) with collagenase, it became possible to prepare

intact liver cells in high yield. By this method it is possible to convert most of the liver into a suspension of intact cells. Various methods for the preparation of rat hepatocyte suspensions have been compared with regard to cell yield and viability index (34).

Zahlten et al. (35) discovered a simple modified method for hepatocyte isolation which could be performed in any laboratory without a temperature controlled perfusion chamber. Recently, Gustavsson (36) reported that liver cells prepared by collagenase perfusion were better than liver cells prepared from slices. They had a higher viability, RNA and protein content, and initial O_2 consumption. The rates of protein degradation and synthesis were also higher. These observations support the claim that the collagenase perfusion method is better than all previous techniques. Collagenase coupled with very mild mechanical treatment is the method of choice.

The development of techniques for the preparation of liver cell and some uses of these cells are discussed in detail in "Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies" (37).

The Use of Isolated Liver Cells

Hepatocytes have been isolated from various animal livers, including dog, cat, rabbit, rhesus monkey, chicken, guinea pig, mouse and rat. However, most investigators to date have used rat liver as the source of hepatocytes. The isolated hepatocyte has been widely used to study carbohydrate, protein, lipid and nucleic acid metabolism, cancer, drug metabolism, biochemical pharmacology, and immunology (See Appendix).

It has been demonstrated that the carbohydrate metabolism of

isolated liver cells closely resembles that of intact perfused liver (7, 10, 33, 37-41). Carbohydrate metabolism of isolated hepatocytes has been studied by many investigators. These studies include gluconeogenesis (30, 39, 40, 42-46), glycogen synthesis (47-50, 53) and degradation (43), glycolysis with either glucose (47) or fructose (33, 50) as substrate, futile cycles (51, 52), and glycolytic and gluconeogenic enzymes (54-56).

As mentioned before, the nutritional state plays an important role in hepatic metabolism. Meal fed (8+ 16 hr cycle) (47, 48), ad libitum fed, and 24, 48 or 72 hr fasted rats were the animals of choice for most reported studies.

Isolated hepatocytes from fasted rats have a low ability to utilize glucose at the physiological concentration range (5-10 mM glucose); 20 mM glucose or more is needed to observe net glycolysis and glycogen synthesis (47, 48, 50, 51, 57, 58). Hepatocytes from fasted rats have a rapid rate of gluconeogenesis from lactate, pyruvate, fructose and amino acids. Fructose is the best glucogenic substrate, the formation of glucose being 270% greater than that from lactate (30, 38, 50, 59). The gluconeogenic capacity (36, 44, 51, 60, 61) is greater than that in hepatocytes from fed rats. However, fasted hepatocytes have a lower glycogen synthesis capacity compared to cells from fed rats (47, 50). Hepatocytes from fed rats formed glycogen at a rate of 42 μmole/g/90 min as compared to cells from fasted rats which formed glycogen at a rate of 2 μmole/g/90 min (47). Fructose is utilized very effectively by isolated hepatocytes from both fed and fasted rats (50). Studies with cells from fasted rats clearly agree with the known gluconeogenic role of liver. Under hypoglycemic conditions glucose accumulates in the

medium (incubation or perfusion), while under hyperglycemic conditions a net consumption of glucose occurs (7-10, 50, 62, 63).

Hepatocytes from fed rats took up more glucose than those from fasted rats (52). There was a great increase of fatty acid synthesis in cells from meal fed as compared to cells from ad libitum fed rats (57).

It is well known that liver contains four types of glucose-ATP 6-phosphotransferase. Three of them, the hexokinases (E.C. 2.7.1.1), are non specific for hexose and have a high affinity for glucose (Km of 10^{-4}-10^{-6} M). The fourth one is glucokinase (E.C. 2.7.1.2) which has a high Km of 10^{-2} M. The hexokinase activity is unchanged upon starvation while glucokinase is decreased (50, 54, 64-66). Whether or not both hexokinase and glucokinase are present in the isolated liver parenchymal cell was studied by Werner et al. (54). They confirmed that isolated hepatocytes have both glucokinase and hexokinase. The hexokinase activity in the isolated liver cell is about half of that found in whole liver homogenates (54). When rats are starved for 1 to 7 days, there is no significant change in hexokinase activity in both isolated hepatocytes and whole liver homogenates whereas glucokinase activity is greatly decreased. Whole liver homogenates from fed rats have hexokinase at a level of 0.43 and glucokinase 1.39 U/g wet wt., whereas hepatocytes have hexokinase at a level of 0.23 and glucokinase 1.50 U/g wet wt. (54). After a 24 hr fast whole liver homogenates have hexokinase at a level of 0.38 and glucokinase 0.61 U/g wet wt.; hepatocyte have hexokinase at a level of 0.25 and glucokinase 0.54 U/g wet wt. (54).

The Glc-6-P, Fru-6-P concentration in hepatocytes isolated from meal fed rats (3 hr after a meal) and incubated 1 hr in a medium

containing 16.5 mM glucose was 0.689, 0.245 nmole/mg dry weight, respectively (67). The Glc-6-P concentration of hepatocytes, reported by Katz (52), was 0.1 mM.

The Significance of Mannose Metabolism

Mannose is an important component of glycoproteins and glycolipids. The presence of mannose and fucose in the carbohydrate moiety of various glycoproteins is well known (68). It is the major carbohydrate component of glycoprotein in rat brain (69-71). Mannose does not normally constitute an important part of an animal diet. However, it can serve as a major energy substrate for perfused rat brain, substituting for glucose (72).

In mammalian systems mannose is phosphorylated by hexokinase and glucokinase to mannose-6-phosphate (Man-6-P) (73, 74), which enters glycolysis via fructose-6-phosphate (Fru-6-P), by the action of phosphomannose isomerase (PMI) (75, 76). The PMI was first reported by Slein (75-77) to be different from phosphoglucose isomerase (PGI). PMI of yeast (78-81) and konjak corms (82, 83) have been thoroughly studied. These purified PMIs are similar in stability, molecular weight and some kinetic properties. However, the mammalian PMI has not been well characterized.

PMI activity in various mammalian tissues are found at a level of 1 to 4 units/g wet wt. of tissue (84). One function of PMI in mammalian tissue is to catalyze the interconversion of Man-6-P and Fru-6-P, providing an entry to glycolysis for mannose. Mammalian tissue probably has two pathways of mannose metabolism, one leading to glycolysis, the other to glycoprotein synthesis via GDP mannose, the precursor of

mannose in glycoproteins and of the important glycoprotein component, fucose. It is assumed that Man-6-P formed from Fru-6-P is converted to GDP-Man via Man-1-P. The synthetic pathway was found in yeast (85), bacteria and algae (86, 87).

How Man-6-P is converted to Man-1-P in mammalian systems is still unclear. Whether or not mammalian tissues has a unique phosphomannomutase (PMM) is not known. Lowry and Passonneau (88) found that phosphoglucomutase (PGM) from rabbit muscle, which interconverts Glc-6-P and Glc-1-P, also catalyzes the interconversion of Man-6-P and Man-1-P. However, the reaction is much slower than that for Glc-6-P and Glc-1-P. The PGM has a Km and Vm for Glc-1-P and Glc-6-P of 8, 47 μM and 328, 115 μmole/mg/min, whereas for Man-1-P and Man-6-P they are 245, 500 μM and 19.4, 0.2 μmole/mg/min, respectively (88).

In 1979, Asikin and Koeppe (89) reported the concentrations of Man-6-P and Man-1-P in rat brain, kidney, and liver; the average values, respectively, are 51, 21, 99 nmole/g for Man-6-P, and 13, 12, 15 nmole/g for Man-1-P. They also reported a striking increase of Man-6-P in rat liver after intraperitoneal injection of 20 mmole/kg body weight of mannose. Liver Man-6-P increased to 0.4-4.3 μmole/g, and Man-1-P was raised to 100-186 nmole/g. This is the first report of increases in Man-6-P and Man-1-P concentrations with mannose administration.

Purpose of Present Study

Our interest in studying mannose metabolism in isolated rat hepatocytes was stimulated by the report of Asikin and Koeppe (89). The purpose of this study was to find a way to increase the Man-6-P

level in hepatocytes. Success would indicate that hepatocytes might be a good biological system in which to study the interconversion of Man-6-P and Man-1-P.

It is well known that the nutritional state of an animal plays an important role in hepatic carbohydrate metabolism. Katz (52) reported a difference in uptake of glucose by hepatocytes from fed and fasted rats. Therefore, in this study we used hepatocytes from animals in three different nutritional states. These were standard fed (fed ad libitum), 15% mannose fed and 24 hr fasted. Also, it seemed likely that different results would be obtained before and after incubation of hepatocytes with mannose.

The present study was carried out to determine Man-6-P, Glc-6-P and Fru-6-P concentrations in freshly isolated hepatocytes and in those incubated with and without various concentrations of mannose. Organ perfusion was also studied. Rat livers of standard fed, 15% mannose fed, and 24 hr fasted rats were perfused with and without mannose and the concentrations of hexose-6-phosphate were determined. Incubation of hepatocytes with mannose or perfusion of liver with mannose caused a striking increase of Man-6-P concentration, suggesting that hepatocytes and perfused liver might be useful system for studying the formation of Man-1-P and GDP-man.

CHAPTER II

MATERIALS AND METHODS

Materials

Perchloric acid was from J. T. Baker; ATP, NADP, NADH, Glc-6-P, Fru-6-P, Man-6-P, mannose, TEA, TES, HEPES and tricine were from Sigma Chemical Co.; Collagenases, type II; CLSII, Lot #40N072 (154 U/mg); CLSII, Lot #40P162P (138 U/mg); CLSII, Lot #40S174 (181 U/mg); were from Worthington Biochemical Co.; PMI was from Boehringer Mannheim Biochemicals; other enzymes were from Sigma; Charcoal (Norit A) was from Matheson; trypan blue dye solution was from Grande Island Biological Co.; the Nitex Nylon cloth was from Tetko, Inc.; and Prosil-28 was from PCR Research Chemical Inc. All other chemicals were reagent grade or highest purity available. Deionized water obtained from passing the reverse osmosis water through an ion exchange column from SyBron, Barnstead was used for the preparation of reagents and solution. All solutions, except enzymes, used in fluorometric assay were filtered through 45 μm Millipore filter prior to use. Cornwall syringes, plastic disposable syringes, Eppendorf pipettes and Pipetmen were used for transfers. Lens paper was used instead of "Kim Wipes".

Methods

Animals

Adult female Cobs-Outbred rats (Charles River Laboratories), 250-350 g were maintained on Purina laboratory chow ad libitum or 24 hours fasted, or fed on 15% mannose mixed with blended chow for a week. The mannose-fed rats received their food from a container placed on the cage floor, whereas the control rats received their food from a wire top bucket (90).

Glassware

All glassware used in the isolation of hepatocytes were siliconized with Prosil-28 as follows: immerse the cleaned glassware in the Prosil-28 working solution (dilute 1 part of Prosil 28 into 100 parts of water) for 30 seconds, ensuring that all surfaces to be coated come into contact with the solution. Remove from the solution and rinse thoroughly with water to remove any excess of Prosil-28 from the surface. To ensure optimum performance, air dry for 24 hr at room temperature or heat in a 100^{o}C oven for 10 min.

Perfusion Medium

Four kinds of buffer solutions were used in the perfusion technique (91). The composition of each buffer solution is shown in Table I.

Perfusion Apparatus

A simple perfusion apparatus, modified from Seglen's (91), as

TABLE I*

COMPOSITION OF BUFFERS FOR LIVER PERFUSION**

	Solution I	Solution II	Solution III	Solution IV
NaCl	141.9	66.7	141.9	68.4
KCl	6.7	6.7	6.7	5.4
$CaCl_2$	-	1.2	1.2	1.2
$MgCl_2$	-	-	-	0.6
KH_2PO_4	-	-	-	1.1
Na_2SO_4	-	-	-	0.7
HEPES	10.0	100.0	10.0	30.2
TES	-	-	-	30.1
Tricine	-	-	-	36.3
10 M NaOH***	0.55	6.60	0.55	5.25
pH	7.4	7.6	7.4	7.6

*Each solution in this table was gassed with mixture of 95% O_2-5% CO_2 for 15-20 min before use.

**Salt concentrations are given as mM.

***Amount of NaOH added as ml per 1000 ml of final solution.

shown in Figure 1, was used. It consisted of (1) the water jacketed coil tube (a 3 mm diameter glass tube with 15 coils; outer tube diameter 25 mm; coil length 125 mm) which maintains the temperature of the perfusate at 37°C; (2) a combined unit of filter and bubble trap which has a cotton plug filter. The other end of the coil tube is connected by silicon rubber tubing through the pump (3) to the buffer reservoir (specially made by Mr. W. M. Adkins). The water jacketed coil tube is clamped to a flexible holder. The assembly of coil tube-filter-cannula can be moved freely in all directions, which facilitates exactly positioning of the portal cannula and easy transfer of cannulated liver from the body to the liver dish (4). The liver dish was made (by Mr. Adkins) by putting a conical outlet at the bottom of a glass petri dish. A stainless steel net (5) is placed in the liver dish. The perfusate reservoir (6) is made from a 125, 500 or 1000 ml Erlenmeyer flasks with one or two outlets set 90 degrees apart at the bottom of the flasks. One outlet is connected to tubing that leads the perfusate buffer to the liver, the other is an inlet for oxygenation with a mixture of 95% O_2-5% CO_2. When the perfusate is recirculated, the liver dish is placed on a tripod which stands over the reservoir. Silicone rubber tubing is used for all tubing connections. The peristaltic pump by Büchler is capable of producing flow rates up to 35 ml/min using a 3 mm diameter silicone tubing. When non-recirculating perfusing is used, the continuous oxygenation process is omitted and single outlet reservoir is used.

Liver Cell Preparation

In order to minimize the diurnal variation in metabolic processes,

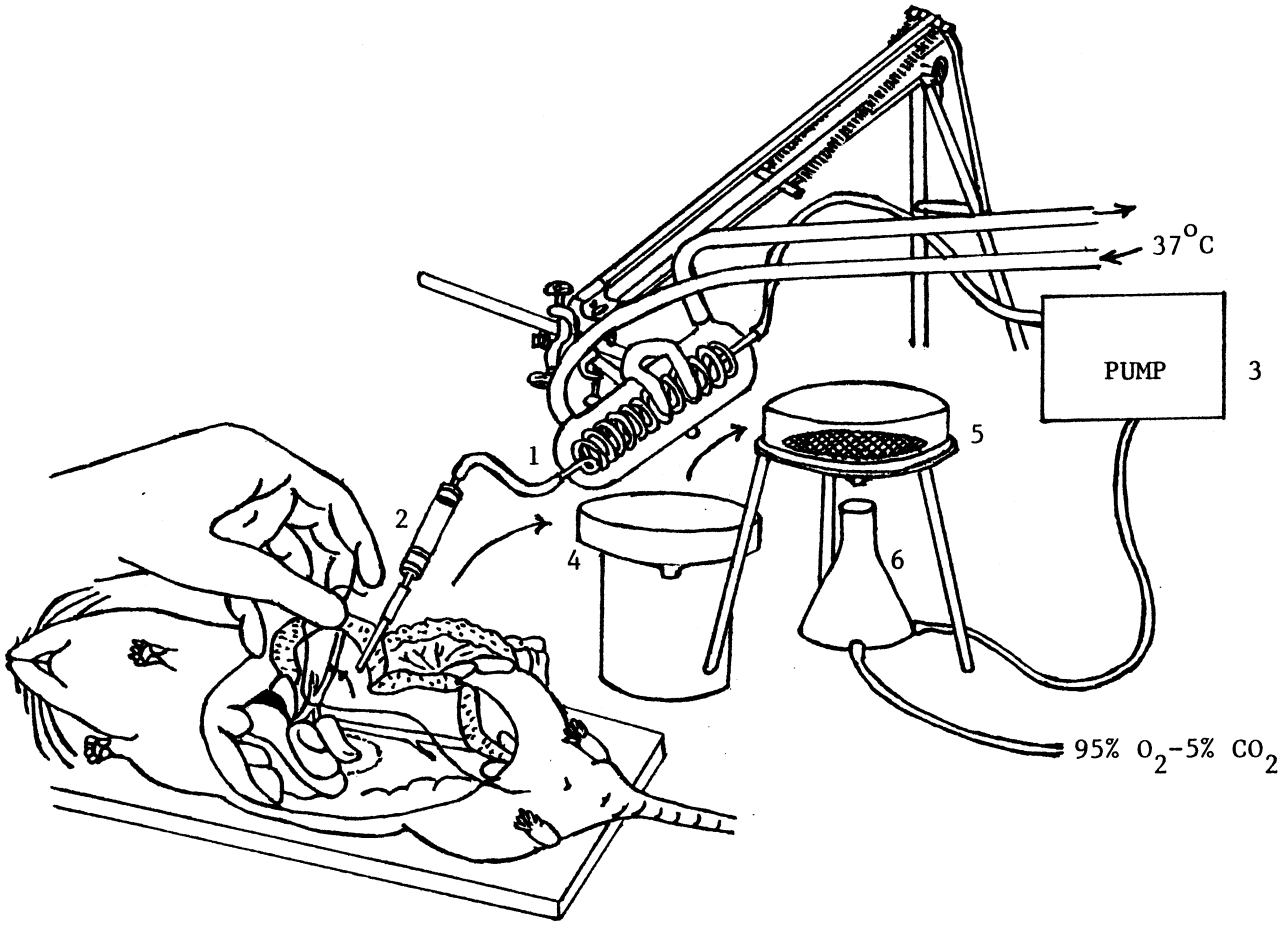

Figure 1. Experimental Arrangement for Isolation and Perfusion of Rat Liver. (1) Water jacketed (37°C) coiled tube; (2) filter and bubble trap; (3) Büchler peristaltic pump; (4) liver dish; (5) stainless-steel net; (6) perfusate reservoir.

hepatocytes were always prepared between 1:00 and 3:00 PM (92, 93), using the following procedure.

The rat was placed in a big jar (5-liter dessicator) with paper towel on the bottom and ether was poured onto a thick layer of cheesecloth that hung from the lid. The lid was opened just enough so the animal could not escape. As soon as the rat became unconcious, usually it took 2-2½ min.; it was rapidly transferred to the operating table (an operating board placed on an enamel tray). Very light anesthesia was maintained by placing the opening of a 50 ml beaker, containing a layer of cheesecloth saturated with ether, in front of the rat's nose. With the rat on its back, the legs were tied to the operating board. The abdomen was opened by a U-shaped transverse incision and the intestines were displaced on the left side of the abdominal cavity. A loose ligature was placed around the vena porta as shown in Figure 1. The slow pumping of perfusate of solution I was started. A nick on the vena porta (portal vein) was made by an 18 gauge needle and cannula tubing with a 45^{o} point was rapidly inserted into the vein and pushed until the tubing was about 5 mm inside the ligature. The ligature was tied tightly and secured with an extra knot. The upper vena cava (between liver and diaphragm) and the inferior vena cava (for perfusate efflux) were cut and perfusate flow increased to 35 ml/min. The first 2-3 min. of perfusion was performed *in situ*. While perfusion of solution I was continued, the liver was removed from the carcass by cutting out other tissues and organs attached to it. The liver was placed, in a position similar to that *in situ*, on the liver dish which was temporarily on top of a 250 ml beaker. When most of solution I (about 500 ml) had been used, the liver should have a uniform

light-tan color with no swelling. Then the system was switched to a recirculation unit as follows: The second reservoir, with double outlet, contained 50 ml of solution II (0.050-0.075% w/v of collagenase) with continuous oxygenation by 95% O_2-5% CO_2. The liver dish was placed on top of the tripod over the reservoir. During the time it took to move the liver dish to this new reservoir, most of solution I in the tubing and filter were flushed out. Subsequent efflux perfusate was returned to the reservoir. The perfusate flow was at maximum, 35 ml/min. The liver was perfused for 20 min with recirculating collagenase buffer (continuously oxygenated) during which time it swelled to more than double its original size (see Discussion). Then the liver was transferred to a petri dish containing 50 ml of cold solution IV. While held by a forceps, it was gently shaken and the cells liberated from the connective vascular tissue by very gentle raking with a plastic comb (2 mm between teeth). When perfusion was perfect, a pale pink gelatinous suspension of connective tissue and vascular tissue was removed by filtration through a coarse (250 μm mesh opening) nylon cloth.

Purification of Hepatocytes

The initial cell suspension obtained by collagenase perfusion contains not only parenchymal cells but also non-parenchymal cells, damaged cells, cell clumps and subcellular debris. These contaminants are removed by a combination of filtration and differential centrifugation as follows. The initial cell suspension from 2 preparations were combined (the second rat started 40-45 min after the first rat was perfused) and preincubated in a large petri dish in a 37°C waterbath

for 10 min with very gentle shaking. Then the petri dish was chilled on ice and the cell suspension was filtered by using a series of diminishing mesh nylon cloth squares. In order to avoid filtration pressure, the cell suspension was slowly poured into the plastic cylinder, which has a nylon cloth attached at one end, placed in the petri dish. As seen in Figure 2, the level of fluid inside the cylinder should only be slightly higher than that outside. The cylinder and filter were slowly lifted through the suspension with continuous gentle shaking. This was repeated through a total of 4 filters, the first two of 100 μm and then two of 64 μm.

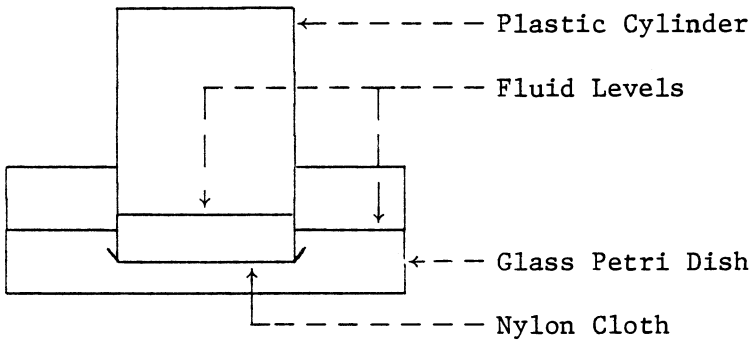

Figure 2. Filtration of Cell Suspension with Nylon Cloths

Such use of a series of filters prevents overloading of a single filter, which might result in excessive filtration pressure damaging the cells squeezed through. The cells were centrifuged in an SS-1 Sorval rotor at 500 rpm for 2 min in the cold room. The supernatants were aspirated and discarded. Then the cells were very carefully

resuspended by swirling in 4 x 40 ml of ice cold solution III and centrifuged again at the same speed. This washing procedure was repeated twice (total of four sedimentations). The final pellet was resuspended in 30-35 ml of solution IV and filtered through a single nylon filter (64 μm mesh). Later, this final cell suspension was adjusted to the desired cell concentration. A sample of the cell suspension was taken for determination of protein content. The final cell suspension was completely free of broken connective tissue, red cells and Küpffer cells as checked under light microscopy (400X).

Trypan Blue Dye Exclusion Test and Cell Count

The most common criterion of cell viability is the trypan blue dye exclusion test. Cells with intact plasma membranes exclude dyes, whereas damaged cells become stained (94). The cellular integrity of preparations was examined by light microscopy for exclusion of trypan blue dye. A differential count of cell preparations was made by diluting 100 μl of cell suspension with 300 μl of 0.6% dye solution and counting in a Bürker chamber (hemocytometer). The cover-glass is first mounted on the chamber. Then the cell suspension is applied at the edge of the coverglass from where it is drawn into the chamber by capillary action.

Incubation of Cell Suspensions

The purified hepatocytes were suspended in solution IV which served as the incubation medium. The great surface and shallow depth of the incubation chamber is necessary to insure sufficient oxygenation. A 3.0 ml sample of hepatocytes (approximately 1.4×10^7 cells/ml) was

incubated in a siliconized vaccine vial with various concentrations of substrate as follows: 10 mM mannose; 20 mM mannose; mixture of 5 mM mannose and 5 mM glucose. Each vial was gassed with 95% O_2-5% CO_2 for 1 min and tightly capped. All vials were incubated in a slowly shaking waterbath at 37°C for 1 hour. Each set of experiments had 2 kinds of controls. One is called "Zero (0) time" control, the other is "Experimental" control. The 0-time control is the final cell suspension. The experimental control is like other incubated samples except no substrate is added. At the end of the incubation time, metabolic reactions were rapidly stopped by adding the deproteinizing reagent, 2.0 ml of 8% perchloric acid-40% ethanol (PCA-EtOH) (95). Then the sugar phosphates were extracted.

Hexose-6-Phosphate Extraction

The incubation mixture of each vial, after mixing with PCA-EtOH and standing in an ice bath for 10-15 min, was transferred to a Potter-Elvehjem vessel and homogenized using six passes of a Teflon pestle driven at 425 rpm. 1-2 drops of PCA-EtOH was added to the vial to transfer the residue to the homogenizer. The homogenate was transferred by Pasteur pipette into a round bottomed centrifuge tube and centrifuged at 10,000 x g for 30 min in the Sorval SS-1 rotor in the cold room. The supernatant was transferred to another graduated centrifuge tube and the yellowish color which is highly fluorescent removed by treatment with 15 mg/ml of activated charcoal (96, 97). This mixture was centrifuged and the supernatant was filtered through a 45 μm Millipore filter and was neutralized to pH 6.0-6.5 by slowly adding 3 M K_2CO_3 containing 0.5 M TEA (95). Potassium perchlorate was

allowed to precipitate for 30 min in an ice bath and removed by centrifugation at 10,000 x g for 30 min. The neutralized extract was concentrated by rotatory evaporation (< 30°C) to less than 1 ml and kept on ice in the refrigerator until sugar phosphate and free sugar were measured. The recovery of Glc-6-P was determined on each test of every experiment, as follows. There were duplicate vials for each test in a given experiment. After adding PCA-EtOH to stop metabolic reaction, the two vials of the same protocols were combined and homogenized together. This homogenate was divided equally between two tubes and a known amount of Glc-6-P (20 nmoles) was added to one of them. These two tubes were extracted as above.

Assay of Metabolites

The metabolites were determined on a Johnson Research Foundation Metabolic Fluorometer. The fluorometer was supplied with a low pressure mercury lamp and excitation filter with maximum transmission at 355 nm and 53 nm half width. On the emission side filters were used to exclude the wavelengths that were below 420 nm or above 500 nm (98). The free hexose was determined spectrophotometrically in a Gilford Spectrophotometer Model 240 connected with a Digital Absorbance Meter Model 410. The Glc-6-P, Fru-6-P, and Man-6-P were determined via enzymatic assay using a coupled reaction of glucose-6-phosphate dehydrogenase (G-6-PDH) as shown in Figure 3; the increase in absorbance or fluorescence of NADPH were measured (95, 99).

All assays were carried out in 0.1 N TEA buffer pH 7.6. In a final volume of 2 ml, the indicated of the following reagents were added: NADP, 400 μM; PMI (yeast) 1.5 U; PGI (yeast) 1.25 U; and

G-6-PDH (yeast) 1.25 U. The assay mixture was preincubated for 5 min at 37°C in the incubation chamber, then it was transferred to the reading chamber. The reaction was initiated by addition of 5 µl (equivalent to 1.25 U) of G-6-PDH. The Glc-6-P, Fru-6-P, and Man-6-P were assayed by sequential addition of G-6-PDH, PGI and PMI to the assay mixture. Since some batches of PMI contained PGM activity, PGM (rabbit muscle) 1 U was added before PMI to remove any Glc-1-P present. The PMI reaction was complete in 20-30 min. In order to reduce assay time, it was recommended by Asikin (100, 101) that the first cuvette be removed after adding PMI, and placed in the incubator chamber of the fluorometer while starting another reaction. After the addition of PMI to a third reaction, the first cuvette is returned to the reading chamber and the final fluorescence read.

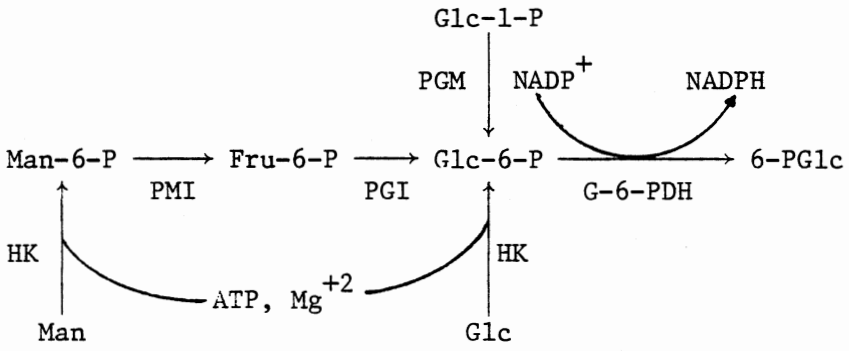

Figure 3. Schematic for Enzyme Coupling System

Free mannose and glucose were assayed spectrophotometrically in the presence of ATP, 300 µM; $MgCl_2$, 3 mM; HK 1.5 U with and without

addition of PGI and PMI. The glucose was measured by the addition of HK after the measurement of Glc-6-P. The addition of PGI gave Fru-6-P and the addition of PMI gave mannose and Man-6-P. The free mannose was obtained by subtracting the Man-6-P reading in the absence of HK from those in its presence.

The sample size of this study was adjusted so that the total metabolites being sequentially assayed was not more than 20 nmoles/2 ml.

The concentration of metabolites was calculated based on molar absorbance of NADPH of 6.22×10^6 $cm^{-1}M^{-1}$. The fluorometer was standardized by determining the fluorescence developed using a known concentration of glucose or Glc-6-P or NADPH. The data presented in this study are taken from a duplicate measurement and corrected for the recoveries in each sample.

Isolated Liver Perfusion

The liver was first preperfused *in situ* and *in vitro* with solution I, 500 ml, to remove blood and vascular metabolites and then was perfused for 1 hr *in vitro* with a non-recirculating solution IV 10 mM in mannose. The perfusates were collected for determination of free hexose and hexose-6-phosphate. At the end of 1 hr the liver was perfused with another 50 ml of solution IV to flush out any residual mannose, rapidly frozen in acetone-dry ice, and the metabolites were extracted as described above for hepatocytes, using a slight modification of Asikin's procedure (89, 100). The metabolites of liver perfusion were assayed as described above.

Protein Determination

Protein concentrations were estimated by the method of Lowry et al. (102), using bovine serum albumin as standard.

CHAPTER III

RESULTS

Prior to performing experiments at OSU, Dr. H. O. Spivey and I visited Dr. J. A. Ontko at the Oklahoma Medical Research Foundation to observe his method for the isolation of hepatocytes. Later, I learned and practiced the method for isolating hepatocytes with Dr. J. M. Merz, who is well-trained in organ perfusion. The method of isolation of hepatocytes used in this study was a slight modification of that described by Seglen (91).

Many practice experiments were done to improve our skill in obtaining good cell preparations. Preliminary experiments were run in order to find the best collagenase concentration and an adequate perfusion time. Based on the data presented in Figure 4 and Figure 5 a 0.05% collagenase solution (see Discussion) and a 20 min period of perfusion time were chosen for this study.

I found that good cell preparations were obtained when I could observe a liver swollen to double the original size after collagenase perfusion (see Discussion). Any cell preparations that contained more than 87% viable parenchymal cells were used for mannose metabolite studies.

Viable cells were determined by the trypan blue dye exclusion test. Whenever the plasma membrane of a hepatocyte permits entry of dye, there is probable loss of soluble enzymes which would make such

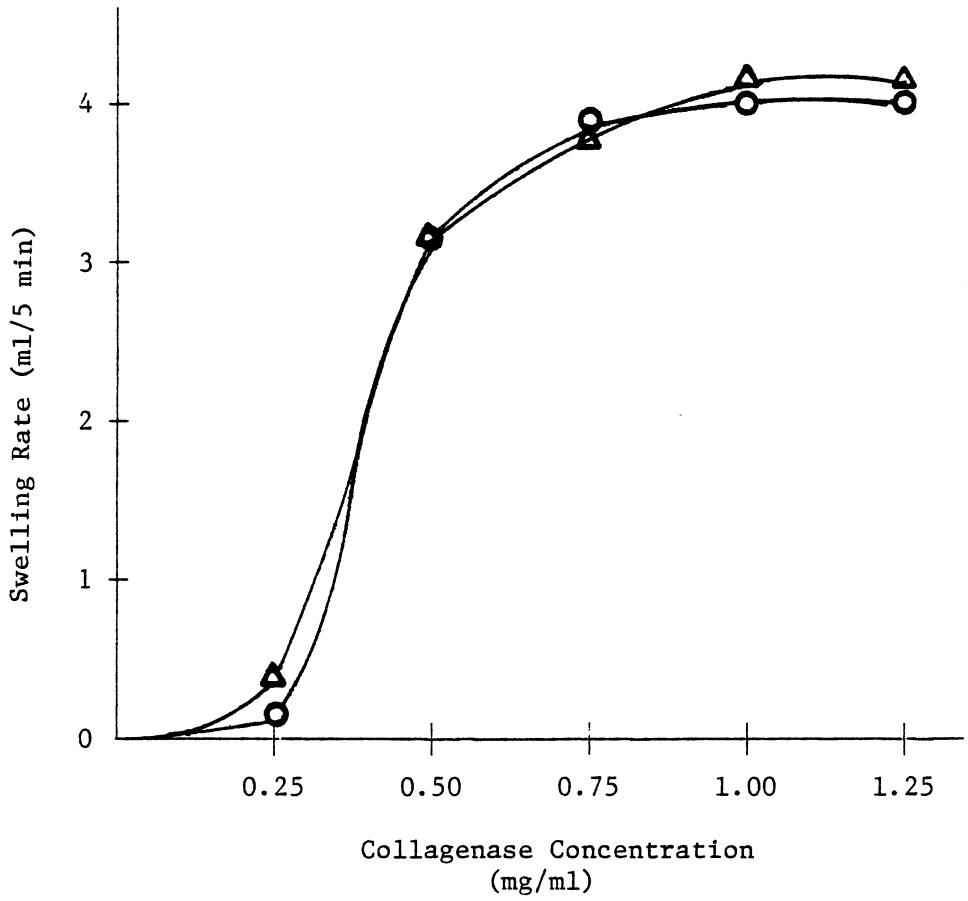

Figure 4. Effect of Collagenase Concentration on Liver Swelling

Data are obtained from two rats (o, Δ).

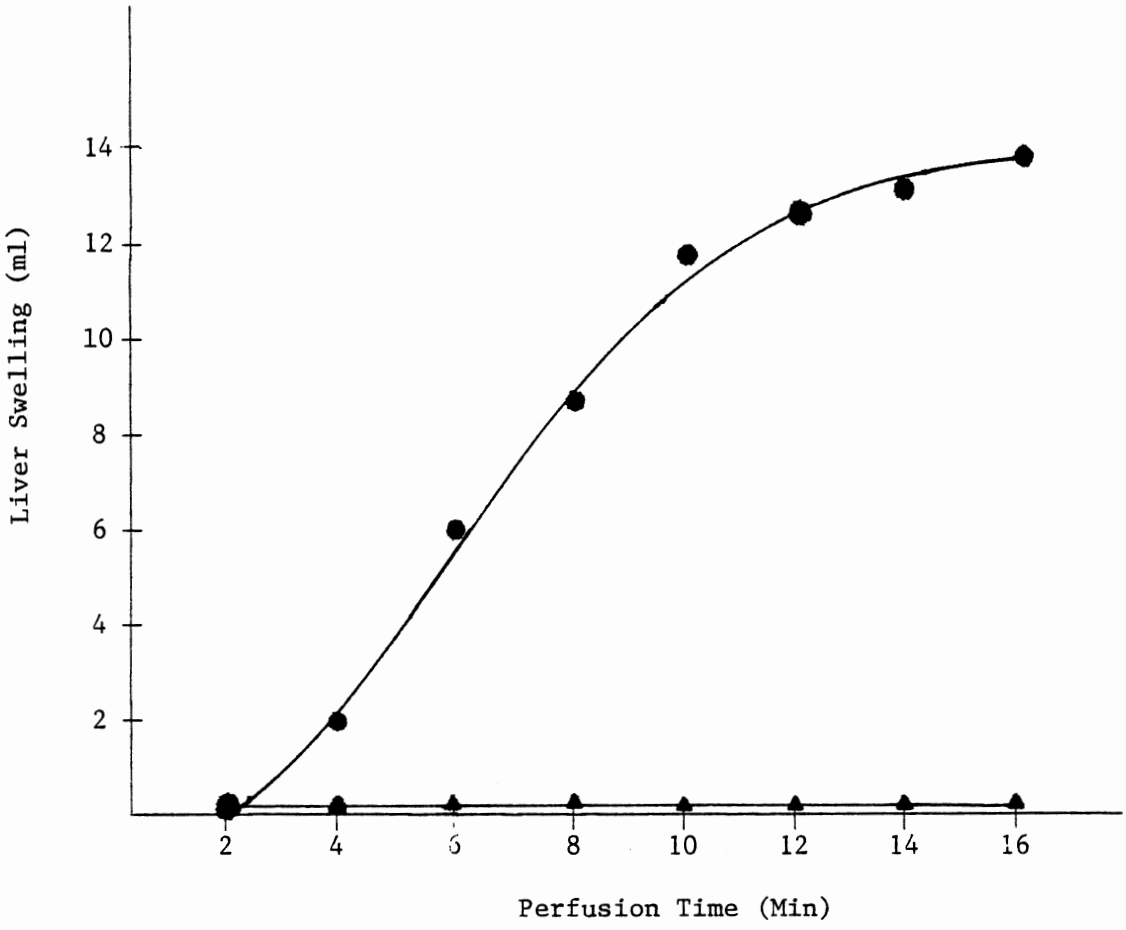

Figure 5. The Rate of Swelling During Collagenase Perfusion

●——● Liver swelling during 0.05% collagenase perfusion.

▲——▲ Liver swelling during perfusion without collagenase.

a cell incapable of normal cellular activity (103). The viability of hepatocytes of initial and final cell suspensions was checked with trypan blue as shown in Table II. There were no differences in cell viability due to nutritional status (see Discussion). Purification did not increase the percentage of viable cells, but did decrease the percentage of non-parenchymal cells and the amount of subcellular debris. The final cell suspensions are 90% viable parenchymal cells and 3-4% non-parenchymal cells. The yields, wet weights, protein concentrations, and the numbers of hepatocytes are presented in Table III.

In this study the concentrations of hexose-6-phosphate were corrected for recoveries, which varied between 84 and 110%.

Hexose-6-phosphate concentrations were determined for freshly isolated hepatocytes from various rats: standard fed, 15% mannose fed and 24 hr fasted (Table IV). It is found that the levels of hexose-6-phosphate in isolated hepatocytes from starved andimals are lower than those from fed animals. The Glc-6-P, Fru-6-P and Man-6-P of freshly isolated hepatocytes from fasted rats are 37%, 37.5% and 42.9%, respectively of those from fed rats. There is no difference in hexose-6-phosphate levels between cells isolated from standard fed and mannose fed rats.

Hexose-6-Phosphates in Hepatocytes Incubated with Mannose

Not only cells isolated from standard fed animals, but also cells isolated from mannose fed and fasted animals were studied. Isolated hepatocytes were incubated in 10 mM mannose, 20 mM mannose, and a

TABLE II

ISOLATED LIVER CELL CHARACTERISTICS BEFORE AND AFTER PURIFICATION

Rat Condition	Standard fed (6)*		15% Mannose fed (5)		24 hr Fasted (8)	
Sample	viability**	non-parenchymal cell***	viability	non-parenchymal cell	viability	non-parenchymal cell
Initial cell suspension	89.0 ± 1.6	13.5 ± 0.6	89.4 ± 0.9	13.2 ± 0.5	89.6 ± 1.1	14.5 ± 1.1
Final cell suspension	90.3 ± 0.8	3.0 ± 0.6	89.6 ± 0.9	2.6 ± 0.6	90.4 ± 1.1	3.5 ± 0.8

*Number of experiments in parenthesis.

**% of parenchymal cells viable based on trypan blue dye exclusion test (Mean ± S.D.).

***% of total cell count (Mean ± S.D.).

TABLE III

YIELD OF HEPATOCYTES AND CELL PROTEIN*

Rat body weight	278.0 ± 3.0**	g
Liver wet weight	8.6 ± 0.4	g
Yield of hepatocyte wet weight***	6.8 ± 0.3	g
Yield of hepatocyte wet weight****	2.0 ± 0.1	g
Protein of final total yield	460 ± 40	mg
Protein concentration/hepatocyte	226 ± 8	mg/g
Hepatocytes (number of cells)	$(2.6 \pm 0.2) \times 10^8$	

*Experiments were performed on 24 hour fasted rats.

**Values are mean ± S.D.; n = number of experiments = 3.

***Wet weight of initial packed cells.

****Wet weight of final packed cells.

TABLE IV

HEXOSE-6-PHOSPHATE CONCENTRATIONS IN FRESHLY ISOLATED HEPATOCYTES

Rat Condition*	Glc-6-P	Fru-6-P	Man-6-P
Standard fed (n = 6)	0.27 ± 0.02** (0.24-0.29)***	0.08 ± 0.01 (0.06-0.09)	0.07 ± 0.01 (0.06-0.09)
15% Mannose fed (n = 5)	0.27 ± 0.02 (0.24-0.30)	0.08 ± 0.01 (0.07-0.10)	0.08 ± 0.01 (0.05-0.09)
24 hr. fasted (n = 8)	0.10 ± 0.01 (0.09-0.11)	0.03 ± 0.01 (0.02-0.04)	0.03 ± 0.01 (0.02-0.04)

*Rat condition see method.

**Values of hexose-6-phosphate expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

n = numbers of experiments (each experiment is obtained from two rats)

mixture of 5 mM glucose and 5 mM mannose for 1 hr at 37°C. The hexose-6-phosphate concentrations found in hepatocytes from standard fed, mannose fed and fasted animals, respectively, are shown in Tables V, VI and VII.

As seen in Table V, incubation with mannose did not change Glc-6-P or Fru-6-P concentrations compared to those of controls incubated with solution IV without mannose. Although differences are apparent, this increase from control to experimental groups is remarkably small. Similar results were obtained with cells isolated from mannose fed animals (Table VI). If the data in Tables V and VI for Glc-6-P are compared, it is apparent that mannose feeding had very little effect.

Results shown in Table VII indicate that the concentration of Glc-6-P, Fru-6-P and Man-6-P in cells isolated from fasted rats (Table VII) is dramatically lower than in cells from fed animals (Tables V and VI), with or without incubation with mannose.

In Table VIII is presented a comparison of Man-6-P concentrations in hepatocytes, isolated from rats under different dietary conditions, after incubation with various concentrations of mannose (data taken from Tables V, VI and VII). It will be noted that no difference in Man-6-P concentrations is observed between cells incubated in 10 mM or 20 mM mannose. The Man-6-P concentration is increased 3 to 5 fold over that of controls incubated without mannose. This was observed with all hepatocytes. However, in those cells incubated with a lower concentration of mannose, the final Man-6-P level is only 47%, 50% and 73% of those incubated with higher mannose concentration (10 mM or 20 mM) from standard fed, 15% mannose fed and fasted rats, respec-

TABLE V

HEXOSE-6-PHOSPHATE CONCENTRATIONS IN ISOLATED HEPATOCYTES FROM STANDARD FED RAT (n=6*) AFTER INCUBATION WITH VARIOUS CONCENTRATIONS OF MANNOSE

Hexose-6-phosphate	Control (-substrate)	10 mM Mannose	20 mM Mannose	5 mM Glucose 5 mM Mannose
Glc-6-P	0.31 ± 0.03** (0.25-0.35)***	0.34 ± 0.05 (0.27-0.39)	0.34 ± 0.05 (0.26-0.41)	0.31 ± 0.03 (0.24-0.35)
Fru-6-P	0.07 ± 0.01 (0.06-0.08)	0.08 ± 0.01 (0.07-0.09)	0.08 ± 0.01 (0.07-0.09)	0.08 ± 0.01 (0.07-0.09)
Man-6-P	0.07 ± 0.01 (0.06-0.07)	0.30 ± 0.02 (0.27-0.32)	0.31 ± 0.03 (0.27-0.34)	0.14 ± 0.01 (0.12-0.15)

*n = number of experiments; each experiment is obtained from two rats.

**Values are expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

TABLE VI

HEXOSE-6-PHOSPHATE CONCENTRATIONS IN ISOLATED HEPATOCYTES FROM 15% MANNOSE FED RAT (n=5*) AFTER INCUBATION WITH VARIOUS CONCENTRATIONS OF MANNOSE

Hexose-6-phosphate	Control (-substrate)	10 mM Mannose	20 mM Mannose	5 mM Glucose 5 mM Mannose
Glc-6-P	0.33 ± 0.02** (0.31-0.35)***	0.37 ± 0.03 (0.34-0.41)	0.37 ± 0.04 (0.34-0.43)	0.33 ± 0.02 (0.31-0.37)
Fru-6-P	0.08 ± 0.01 (0.06-0.09)	0.08 ± 0.01 (0.07-0.10)	0.08 ± 0.01 (0.07-0.10)	0.07 ± 0.01 (0.06-0.10)
Man-6-P	0.07 ± 0.01 (0.05-0.07)	0.30 ± 0.02 (0.28-0.32)	0.31 ± 0.02 (0.29-0.33)	0.15 ± 0.01 (0.13-0.15)

*n = number of experiments; each experiment is obtained from two rats.

**Values are expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

TABLE VII

HEXOSE-6-PHOSPHATE CONCENTRATIONS IN ISOLATED HEPATOCYTES FROM 24 HOUR FASTED RAT (n=8*) AFTER INCUBATION WITH VARIOUS CONCENTRATIONS OF MANNOSE

Hexose-6-phosphate	Control (-substrate)	10 mM Mannose	20 mM Mannose	5 mM Glucose 5 mM Mannose
Glc-6-P	0.10 ± 0.01** (0.08-0.11)***	0.11 ± 0.01 (0.11-0.12)	0.12 ± 0.01 (0.11-0.12)	0.11 ± 0.01 (0.09-0.11)
Fru-6-P	0.03 ± 0.01 (0.01-0.03)	0.03 ± 0.01 (0.02-0.05)	0.04 ± 0.01 (0.03-0.05)	0.04 ± 0.01 (0.03-0.05)
Man-6-P	0.03 ± 0.01 (0.01-0.04)	0.11 ± 0.01 (0.10-0.12)	0.11 ± 0.01 (0.10-0.12)	0.08 ± 0.01 (0.07-0.10)

*n = number of experiments; each experiment is obtained from two rats.

**Values are expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

TABLE VIII

COMPARISON OF MANNOSE-6-PHOSPHATE CONCENTRATIONS IN HEPATOCYTES ISOLATED FROM RATS ON DIFFERENT DIETS AND INCUBATED WITH VARIOUS CONCENTRATIONS OF MANNOSE*

Animal Condition	Substrate			
	Control (-substrate)	5 mM Mannose 5 mM Glucose	10 mM Mannose	20 mM Mannose
Standard fed	0.07 ± 0.01 (0.06-0.07)	0.14 ± 0.01 (0.12-0.15)	0.30 ± 0.02 (0.27-0.32)	0.31 ± 0.03 (0.27-0.34)
15% Mannose fed	0.07 ± 0.01 (0.05-0.07)	0.15 ± 0.01 (0.13-0.15)	0.30 ± 0.02 (0.28-0.32)	0.31 ± 0.02 (0.29-0.33)
24 hr. fasted	0.03 ± 0.01 (0.01-0.04)	0.08 ± 0.01 (0.07-0.10)	0.11 ± 0.01 (0.10-0.12)	0.11 ± 0.01 (0.10-0.12)

*Values were taken from Tables V, VI, and VII.

tively.

The free hexose concentrations were determined for freshly isolated hepatocytes (Table IX). As expected, the glucose concentration in hepatocytes from fasted rats is much lower than in those of normal or mannose fed rats.

Liver Perfusion Study

The rat liver was perfused at 37°C for 1 hr with solution IV containing 10 mM mannose in a non-recirculating system to maintain a constant substrate concentration passing through the liver. Control livers were perfused with solution IV without mannose. The hexose-6-phosphates were determined and the values corrected for recoveries which varied between 81% and 109%. Data are presented in Table X.

No differences were observed between standard fed and mannose fed livers. Perfusion with 10 mM mannose increased Man-6-P 4 to 5 fold in fed livers but only 2-fold in fasted livers. However, perfusion with mannose had little effect on Glc-6-P or Fru-6-P concentrations compared to controls. In fasted liver the hexose-6-phosphate concentrations are much lower than in fed livers.

Both glucose and mannose concentrations were determined after perfusion. Results are shown in Table XI. The glucose concentrations in controls are similar to those perfused with mannose. With fasted livers, not only are the low glucose concentrations not increased by mannose perfusion, but also mannose concentrations after perfusion are much lower than with fed livers. The mannose found in the liver extracts should be the mannose taken up by cells, not any residual mannose of perfusion.

TABLE IX

GLUCOSE CONCENTRATIONS IN FRESHLY ISOLATED HEPATOCYTES

Rat Condition	Glucose nmole/mg protein
Standard fed (n=6)*	17.3 ± 1.5** (15.0-19.5)***
15% Mannose fed (n=5)	16.4 ± 1.8 (13.5-18.2)
24 hr fasted (n=8)	1.6 ± 0.2 (1.4 - 1.8)

*n = number of experiments; each experiment is obtained from two rats.

**Values are expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

TABLE X

HEXOSE-6-PHOSPHATE CONCENTRATIONS IN PERFUSED LIVER

Rat Condition	10 mM Mannose	Glc-6-P	Fru-6-P	Man-6-P
Standard fed (n=5)*	-	1.51 ± 0.26** (1.10-1.73)***	0.33 ± 0.10 (0.24-0.49)	0.29 ± 0.03 (0.27-0.33)
(n=7)	+	1.88 ± 0.02 (1.62-2.23)	0.38 ± 0.06 (0.30-0.47)	1.46 ± 0.32 (1.05-1.92)
15% Mannose fed (n=4)	-	1.53 ± 0.24 (1.33-1.80)	0.36 ± 0.06 (0.30-0.43)	0.30 ± 0.04 (0.28-0.35)
	+	1.89 ± 0.16 (1.66-2.04)	0.37 ± 0.04 (0.31-0.41)	1.47 ± 0.21 (1.26-1.76)
24 hr fasted (n=4)	-	0.23 ± 0.02 (0.21-0.24)	0.06 ± 0.003 (0.05-0.06)	0.05 ± 0.001 (0.05-0.06)
	+	0.23 ± 0.02 (0.21-0.35)	0.06 ± 0.003 (0.05-0.06)	0.10 ± 0.009 (0.10-0.11)

*n = number of experiments; each experiment is obtained from two rats.

**Values are expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

TABLE XI

HEXOSE CONCENTRATIONS OF LIVER AFTER PERFUSION

Rat Condition	10 mM Mannose	Hexose concentration	
		Glucose	Mannose
Standard fed (n=5)*	-	25.56 ± 2.07** (22.76-27.95)***	-
(n=7)	+	26.48 ± 4.27 (21.99-31.77)	12.03 ± 6.04 (6.22-23.85)
15% Mannose fed (n=4)	-	24.35 ± 3.24 (21.36-27.43)	-
	+	25.02 ± 3.29 (21.38-28.73)	7.88 ± 2.66 (4.63-10.24)
24 hr fasted (n=4)	-	1.07 ± 0.05 (1.03-1.14)	-
	+	1.08 ± 0.06 (1.03-1.13)	0.07 ± 0.04 (0.04-0.13)

*n = number of experiments; each experiment is obtained from two rats.

**Values are expressed as nmole/mg protein (mean ± S.D.).

***Numbers in parenthesis give the range.

It should be noted here that the perfusates after liver perfusion contained no hexose-6-phosphate.

CHAPTER IV

DISCUSSION

Methodology

Intact hepatocytes can be prepared in high yield by perfusion of the liver with collagenase. A successful liver cell preparation requires careful consideration of several factors.

The gentle handling of the animal (100) is important. Rats should be relaxed and calmed since hepatic vasoconstriction occurs in the anxious animal. Also vascular collapse (91) may occur with too deep ether anesthesia. Gradual introduction of ether anesthesia is desirable because rats so treated are less frightened than those put directly into an ether filled tank or given an injection of anesthetic.

The amount of collagenase used for perfusion in these experiments varied between 0.05-0.10% solution. The need for variation is due to the fact that the different batches purchased had different dispersion efficiencies (37, 91, 104). For this study a fresh daily preparation of 0.05% collagenase in 50 ml of solution II was the optimal concentration in most cases.

Ca^{+2} plays an important role in collagenase perfusion. For an effective perfusion the two step procedure with and without Ca^{+2} recommended by Seglen (105) was used. First preperfusion was performed, without recirculation, at a high flow rate with a large volume of a

Ca^{+2} free perfusate to remove Ca^{+2} (it has a role in cellular adhesion (23, 24)) and any trace of blood from the organ. Because collagenase is a Ca^{+2} dependent enzyme (106), Ca^{+2} is included in the second perfusion medium to enhance enzymatic activity and accelerate dispersion (105).

A good cell preparation is obtained when the liver swells to double its original size. This swelling is not due to circulatory failure or swelling of individual cells, since the isolated cells have a normal structure and water content (30). Swelling is caused by the expansion of extracellular space when the collagenaceous intercellular cement is dissolved and the cells move apart (30).

After collagenase perfusion a very mild mechanical force is used to break the connective and vascular tissue to liberate single cells. This is a critical step since mechanical force could damage the cells. This is done by gentle combing with widely spaced teeth (30).

The buffering system during perfusion and incubation is another important factor. A strong buffering system is required to maintain a constant of pH during the perfusion period. This can be accomplished by a pH stat (105, 107), by the CO_2/bicarbonate system (108), or with HEPES at high concentration (30). HEPES was developed by Good (109). It has a molecular weight of 238.3, a pKa of 7.31 at 37°C and is soluble to the extent of 2.25 M at 0°C. It was evaluated as a good buffer (30). Hepatic anoxia is prevented by continuous oxygenation of the perfusate with 95% O_2-5% CO_2.

The flow rate also plays an important role. A flow rate of 25-30 ml/min with oxygen saturated perfusate gives satisfactory results. It was reported that using a flow rate of 5 ml/min in a non-recirculating system results in a satisfactory cell preparation (110-112). However,

in preliminary experiments with a flow rate of 15 ml/min, we obtained unsatisfactory cell preparations.

One of the advantages of cell suspensions when compared to perfused organs or tissue slices is the possibility of taking many homogeneous samples from one preparation. However, isolated liver cells have a tendency to reaggregate. Proper shaking of cell suspensions before transferring and during incubation is very important.

Because liver cells are metabolically active, rapid stopping of metabolic reactions is important. Hems et al. (113) introduced a special tube for the rapid separation of isolated hepatocytes from incubation medium directly into a deproteinizing agent. Later, Sainsbury et al. (114) found the disadvantage of Hems' special tube, namely a loss of glucose, urea, Na^+ and cell water from the cells while travelling to the bottom of the tube. The substances were lost by moving with water unidirectionally out of cell.

In the earlier part of this study, Hems' method was used for separation of isolated hepatocytes from incubation medium. The special tube described by Hems was made by Mr. Adkins. However, attempts to extract metabolites from the pellets were unsuccessful due to a lack of a special instrument to homogenize the pellets at the bottom of the tube. Spinning the cell suspension in a Sorval SS-1 centrifuge was also tried. Results were unsatisfactory because cells ruptured during centrifugation; hexose-6-phosphate was found in both supernatant fluid and pellet. The method finally chosen was the addition of deproteinizing agent to cell suspensions to terminate metabolic reactions. These cell extracts were concentrated by rotatory evaporation ($< 30^{\circ}C$). This method gave a satisfactory result.

Hepatocytes Study

In this study it was found that the viability of cells isolated from fed animals is the same as from fasted animals. However, Dickson et al. (103) reported that cells from fasted animals were more fragile than those of fed animals. Viable liver cells isolated from 24 hr fasted chickens and fed chickens were 66.4% and 75.6%, respectively.

In Table XII is a comparison of the yield, of hepatocytes and cell protein found by others, with those reported herein. In general the present data are close to those previously reported. The close agreement with literature data indicate that the technique of isolation of hepatocytes in this laboratory was effective. Although my final yield of cells was only 25%, this was sufficient for the studies proposed.

Effect of Mannose Diet on the Concentration of Hexose-6-Phosphates

This study demonstrated that a high mannose diet had no effect on hexose-6-phosphate concentrations in either freshly isolated hepatocytes, those incubated with mannose, or liver perfused with and without mannose. Also we have shown (Table XIII) that a prolonged high mannose diet did not induce an increase in PMI in rat liver. It was found that rats maintained on a diet as high as 30% mannose for six weeks showed no significant changes in the PMI activity of their livers (Table XIII).

Sharma et al. (116) reported that in the rat fasted 72 hr and refed with mannose (5 mmole) for 4 hr, the glucokinase level was the same as in the 72 hr fasted rat. However, when refed with glucose instead of

TABLE XII

COMPARISON OF YIELD OF HEPATOCYTES AND CELL PROTEIN TO LITERATURE VALUES

	Presented data*	Geelen (115)**	Seglen (30)
% collagenase	0.05	0.025	0.05
Perfusion time (min)	20	20	10
Rat body weight (g)	278	275	270-310
Liver wet weight (g)	8.6	8.3	-
Yield of hepatocytes wet weight (g)***	6.8 (79.3%)	-	- (83.8%)
Yield of hepatocytes wet weight (g) ****	2.0 (23.9%)	2.0 (24.0%)	- (36.2%)
Dry weight of total yield (mg/g)	-	233	272
Protein of total yield (mg)	460	430	-
Protein/weight hepatocyte (mg/g)	226	216	228
Hepatocytes (numbers of cells)	2.6×10^8	2.4×10^8	1.3×10^8

*Data are taken from Table III.

**Number in parenthesis is reference number.

***Wet weight of initial packed cells.

****Wet weight of final packed cells.

TABLE XIII

LIVER MANNOSE-6-PHOSPHATE ISOMERASE (PMI) LEVELS*
IN VARIOUS DIETARY CONDITIONS

Rat conditions (n)	PMI activity U/g wet weight
Standard chow fed (4)	3.28** (2.84-3.66)***
15% mannose chow fed (4)	3.04 (2.31-3.93)
30% mannose chow fed (4)	3.04 (2.83-3.27)
Standard chow fed (7)****	3.44 (2.93-4.09)
30 hr fasted (4)	3.05 (2.77-3.34)

*PMI determination see methods in MANOCHIOPINIG, S., M.S. THESIS (84).

**Figure are means of duplicate values. n = number of experiments.

***Numbers in parenthesis give range.

****Values are taken from MANOCHIOPINIG, S., M.S. THESIS (84).

mannose, the glucokinase was restored to the level of the fed animal. Clearly dietary mannose has no important role in controlling the hexose-6-phosphate concentrations.

Effect of Starvation on Hexose-6-Phosphate Concentrations in Isolated Hepatocytes and Perfused Livers

A comparison of hexose-6-phosphate concentrations found in this study, to previous literature values, is shown in Table XIV. In this table concentrations are expressed as micromolarities (nmole/g wet weight). These values were obtained by multiplying the data in previous tables, expressed as nmole/mg protein, by 226 (the determined mg of protein per g of hepatocytes) or 170 (the determined mg protein per g of liver).

Freshly isolated hepatocytes from fed rats contain higher hexose-6-phosphate concentrations than those of fasted rats. The Glc-6-P concentration in hepatocytes from fed rats is similar (Table XIV) to those reported by Katz et al. (52). However, the Glc-6-P, Fru-6-P concentrations reported by Cook et al. (67) were higher than those reported herein. Cook's data were obtained from a 16.5 mM glucose containing medium. The Glc-6-P, Fru-6-P and Man-6-P concentrations of hepatocytes isolated from fasted rats, reported herein, are the first such report.

Perfused liver from fed rats also contained higher concentrations of hexose-6-phosphate than those from fasted rats. These concentrations are in the same range as those reported by Asikin (100) and Start et al. (117) (See Table XIV). The Glc-6-P, Fru-6-P of perfused liver from

TABLE XIV

COMPARISON OF HEXOSE-6-PHOSPHATES CONCENTRATIONS (NMOLE/G TISSUE) TO THE LITERATURE

State of Animal	Hexose-6-Phosphates	Present Study				Literature Values								
		Hepatocytes		Perfused Liver		Hepatocytes		Livers						
		(-)Mannose	10 mM (+)Mannose	(-)Mannose	10 mM (+)Mannose	Katz (52)	Cook (67)	Asikin (100)		Start (117)	Hornbrook (118)	Ross (9)	Bruneugraber (12)[1]	
Fed	Glc-6-P	70	77	257	321	100	187[2]	294[3]	523[4]	283	-	-	43[5]	152[6]
	Fru-6-P	16	18	56	65	-	66	72	115	60	-	-	17	46
	Man-6-P	16	68	50	250	-	-	99	1285	-	-	-	-	-
24 hr Fasted	Glc-6-P	23	25	38	38	-	-	-	-	-	82	30[7]	30	-
	Fru-6-P	7	7	10	10	-	-	-	-	36	-	-	13	-
	Man-6-P	7	25	8	17	-	-	-	-	-	-	-	-	-

[1]Values were corrected to nmole/g wet wt based on 272 mg dry wt/g wet wt reported by Seglen (30).

[2]Hepatocytes incubated for 1 hr in 16.5 mM Glucose.

[3]Rapid Frozen Liver.

[4]1 hr after IP mannose injection (20 mmole/Kg body wt).

[5]Perfused with 4 mM Glucose.

[6]Perfused with 25 mM Glucose

[7]Perfused without substrate 85 min.

fasted rats are remarkably lower than those of fed rats and the Glc-6-P concentrations are almost the same as those reported by Ross (9) and Brunengraber (12) (Table XIV). An exact comparison of the present study (fasted data) with those of Start (117), Hornbrook (118), Rose (9), and Brunengraber (12) is difficult, since identical procedures were not used.

However, the present study and those previously reported (9, 12, 117, 118) clearly indicate that starvation has a definite effect on the hexose-6-phosphate concentrations.

Mannose Incubation and Perfusion Studies

Mannose is phosphorylated to Man-6-P by either hexokinase or glucokinase (74, 119-121). The concentrations of hexokinase and glucokinase in liver are 0.7 and 4.3 U/g wet weight, respectively (122). The affinity of hexokinase and glucokinase for glucose and mannose are approximately the same (123). Hexokinase has a Km for mannose of 5×10^{-6} M (121) and glucokinase has a Km for mannose of 5×10^{-2} M (120).

Whenever hepatocytes or isolated liver are exposed to mannose, an increase of Man-6-P concentrations in these tissue is observed with either fed or fasted rats. This increase is no doubt due to the action of glucokinase and hexokinase, since both enzymes are present in isolated hepatocytes (54) and whole liver. However, the final concentrations of Man-6-P in tissue (hepatocytes or perfused livers) from fasted rats is lower than those of fed rats. It was reported by many investigators (54, 74, 116, 123-126) that glucokinase activity is decreased during starvation while hexokinase activity remains the same. This may

account for the lower final concentration of Man-6-P in the hepatocytes and perfused livers from fasted animals.

The present study and the mannose loading study (89, 100) clearly demonstrate a Man-6-P concentration increase after liver exposure to mannose. Yet the PMI activity study showed that liver contains 2.3-4.0 U/g wet wt. (Table XIII), values approximately equal to the combined hexokinase-glucokinase activities (see above). Therefore, the accumulation of Man-6-P should not occur. However, the PMI of yeast (78-81) has a Km of 1.35 mM for Man-6-P; there is no report on purified mammalian PMI. Crude rat liver PMI has been checked and found to have an approximate Km of 0.7 mM for Man-6-P (this was done on 1 experiment). This relatively high Km of mammalian PMI may account for Man-6-P accumulation, since the Man-6-P concentration in hepatocytes and perfused livers (Table XIV) is much lower than the Km for PMI.

Hexose-6-Phosphate Concentrations in Isolated Hepatocytes Versus Isolated Perfused Livers

It should be noted here that the concentrations in fed rat perfused livers are much higher than those found in isolated hepatocytes. This is probably due to the starvation of hepatocytes during isolation and purification, since this process was done without exogenous substrate. However, the hexose-6-phosphate concentrations of hepatocytes and perfused livers from fasted rats are about the same. Because the rat was starved before the experiment, any furhter starvation during hepatocyte processing apparently has little effect.

Also we should note that the literature values (9, 12, 117, 118)

from Table XIV showed that Glc-6-P, Fru-6-P in fed liver is higher than those in fasted liver.

CHAPTER V

SUMMARY

Liver parenchymal cells from standard fed, 15% mannose fed and 24 hour fasted rats were isolated by a slight modification of the procedure of Seglen and incubated with varying concentrations of mannose for 1 hour. Livers from fed or fasted rats were perfused for 1 hour, with or without 10 mM mannose as substrate. Glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate concentrations were determined via an enzymatic assay coupled to the formation of NADPH, the fluorescence of which was measured.

Freshly isolated hepatocytes from standard fed or 15% mannose fed rats contain similar amounts of glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate (61, 18, 18 μM, respectively), whereas hepatocytes from 24 hour fasted rats have much lower concentrations (23, 7, 7 μM, respectively). A 1 hour incubation without substrate did not change the hexose phosphate concentrations of these hepatocytes. Livers from fasted rats, perfused without substrate, have much lower hexose-6-phosphate concentrations compared to perfused livers from fed rats (glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate concentrations from fasted rats are 38, 10, 8 μM, respectively, and from fed rats are 257, 56, 50 μM, respectively).

Isolated hepatocytes or livers were exposed to mannose via incubation in 10 mM or 20 mM mannose for 1 hour at 37°C, or perfusion with

10 mM mannose for 1 hour. The incubation or perfusion with mannose has very little effect on glucose-6-phosphate and fructose-6-phosphate concentrations. However, the mannose-6-phosphate concentration is increased 3 to 5 fold (2 fold in fasted perfused) over that of the controls incubated or perfused without mannose. The mannose-6-phosphate concentrations found after exposure to mannose are: fed hepatocyte, 68 μM; fasted hepatocyte, 25 μM; fed perfused liver, 250 μM; fasted perfused liver, 17 μM.

A prolonged high mannose diet (30% mannose for 6 weeks) did not induce an increase in phosphomannose isomerase activity in rat livers. The results obtained suggest that hepatocytes and perfused livers may be useful systems for studying the formation of guanosine diphosphate mannose from mannose-6-phosphate.

A SELECTED BIBLIOGRAPHY

(1) von Holt, C., Schmidt, H., Feldmann, H., and Hallmann, I. (1961) Biochem. Z. 334, 524-533.

(2) Baker, N., Shipley, R. A., Clark, R. E., Incefy, G. E., Skinner, S. S. (1961) Am. J. Physiol. 200, 863-870.

(3) Corey, E. L. and Britton, S. W. (1941) Am. J. Physiol. 131, 783-789.

(4) Trowell, D. A. (1942) J. Physiol. 100, 432-458.

(5) Brauer, R. W., Passotti, R. L., and Pizzolato, P. (1951) Proc. Soc. Exp. Biol., N.Y. 78, 174-181.

(6) Miller, L. L., Bly, C. G., Watson, M. L. and Bale, W. (1951) J. Exp. Med. 94, 431-453.

(7) Hems, R., Ross, B. D., Berry, M. N., and Krebs, H. A. (1966) Biochem. J. 101, 284-292.

(8) Schimassek, H. (1963) Biochem. Z. 336, 460-467.

(9) Ross, B. D., Hems, R. and Krebs, H. A. (1966) Biochem. J. 102, 942-951.

(10) Ross, B. D., Hems, R., Freedland, R. A., and Krebs, H. A. (1967) Biochem. J. 105, 869-875.

(11) Soboll, S., Scholz, R., Freisl, M., Elbers, R., and Heldt, H. W. (1976) in Ref. 37, p. 29-40.

(12) Brunengraber, H., Boutry, M., and Lowenstein, J. M. (1973) J. Biol. Chem. 248, 2656-2669.

(13) Daoust, R. (1958) In "Liver Function" (R. W. Brauer, ed.), Am. Inst. Biol. Sci. Washington, D.C., p. 3-10.

(14) Palade, G. E. and Claude, A. (1949) J. Morphol. 85, 35-69.

(15) Harrison, M. F. (1953) Nature (London) 171, 611.

(16) Kaltenbach, J. P. (1954) Exp. Cell Res. 7, 568-571.

(17) Schneider, W. C. and Potter, V. R. (1943) J. Biol. Chem. 149, 217-227.

(18) Longmuir, I. S. and ap. Rees, W. (1956) Nature (London) 177, 997.

(19) St. Aubin, P. M. G. and Bucher, N. L. R. (1952) Anat. Rec. 112, 797-809.

(20) Jacob, S. T. and Bhargava, P. M. (1962) Exp. Cell Res. 27, 453-467.

(21) Anderson, N. G. (1953) Science 117, 627-628.

(22) Schreiber, G. and Schreiber, M. (1973) Sub. Cell Biochem. 2, 321-383.

(23) Gingell, D., Garrod, D. R. and Palmer, J. F. (1970) in "Calcium and Cellular Function" (Cuthbert, A. W., ed.) Macmillan, London. p. 59.

(24) Moscona, A., Trowell, O. A. and Willmer, E. N. (1965) in "Cells and Tissues in Culture" (Willmer, E. N., ed.), Vol. I, p. 19. Academic Press, London and New York.

(25) Easty, G. C. and Mutolo, V. (1960) Exp. Cell Res. 21, 374-385.

(26) Laws, J. O. and Stickland, L. H. (1961) Exp. Cell Res. 24, 240-254.

(27) Pisano, J. C., Filkins, J. P., and Diluzio, N. R. (1968) Proc. Soc. Exp. Biol. Med. 128, 917-922.

(28) Hommes, F. A., Draisma, M. I. and Molenaar, I. (1970) Biochim. Biophys. Acta 222, 361-371.

(29) Hommes, F. A., Oudman-Richters, A. R. and Molenaar, I. (1971) Biochim. Biophys. Acta 244, 191-199.

(30) Seglen, P. O. (1973) Exp. Cell Res. 82, 391-398.

(31) Mandl, I., MacLennan, J. D. and Howes, E. L. (1953) J. Clin. Invest. 32, 1323-1329.

(32) Howard, R. B., Christensen, A. K., Gibbs, F. A., and Pesch, L. A. (1967) J. Cell Biol. 35, 675-684.

(33) Berry, M. N., and Friend, D. S. (1969) J. Cell Biol. 43, 506-520.

(34) Fry, J. R., Jones, C. A., Wiebkin, P., Bellemann, P. and Bridges, J. W. (1976) Anal. Biochem. 71, 341-350.

(35) Zahlten, R. N. and Stratman, F. W. (1974) Arch. Biochem. Biophys. 163, 600-608.

(36) Gustavsson, B. and Morland, J. (1980) Anal. Biochem. 108, 76-82.

(37) Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Tager, J. M., Soling, H. D. and Williamson, J. R., eds.) Elsevier, N.Y. (1976).

(38) Garrison, J. C. and Haynes, R. C., Jr. (1973) J. Biol. Chem. 248, 5333-5343.

(39) Ingebretsen, W. R., Jr., and Wagle, S. R. (1972) Biochem. Biophys. Res. Comm. 47, 403-410.

(40) Berry, M. N. and Kun, E. (1972) Eur. J. Biochem. 27, 395-400.

(41) Zahlten, R. N., Stratman, F. W., and Lardy, H. A. (1973) Proc. Nat. Acad. Sci. 70, 3213-3218.

(42) Ontko, J. A. (1972) J. Biol. Chem. 247, 1788-1800.

(43) Johnson, M. E. M., Das, N. M., Butcher, F. R. and Fain, J. N. (1972) J. Biol. Chem. 247, 3229-3235.

(44) Ingebretsen, W. R., Moxley, M. A., Allen, D. O. and Wagle, S. R. (1972) Biochem. Biophys. Res. Comm. 49, 601-607.

(45) Cornell, N. W., Lund, P., Hems, R. and Krebs, H. A. (1973) Biochem. J. 134, 671-672.

(46) Cornell, N. W., Lund, P. and Krebs, H. A. (1974) Biochem. J. 142, 327-337.

(47) Seglen, P. O. (1973) FEBS Letts. 30, 25-28.

(48) Seglen, P. O. (1973) FEBS Letts. 36, 309-312.

(49) Solanki, K., Nyfeler, F., Moser, U. K. and Walter, P. (1980) Biochem. J. 192, 377-380.

(50) Seglen, P. O. (1974) Biochim. Biophys. Acta 338, 317-336.

(51) Clark, D. G., Rognstad, R. and Katz, J. (1973) Biochem. Biophys. Res. Comm. 54, 1141-1148.

(52) Katz, J., Rognstad, R., Golden, S. and Wals, P. A. (1976) in Ref. 37, pp. 273-282.

(53) Katz, J., Golden, S. and Wals, P. A. (1979) Biochem. J. 180, 389-402.

(54) Werner, H. V., Bartley, J. C. and Berry, M. N. (1972) Biochem. J. 130, 1153-1155.

(55) Bonney, R. J., Walker, P. R., and Potter, V. R. (1973) Biochem. J. 136, 947-954.

(56) Crisp, D. M. and Pogson, C. I. (1972) Biochem. J. 126, 1009-1023.

(57) Clark, D. G., Rognstad, R. and Katz, J. (1974) J. Biol. Chem. 249, 2028-2036.

(58) Rofe, A. M., James, H. M., Bais, R., Edwards, J. B., and Conyers, R. A. J. (1980) Aust. J. Exp. Biol. Med. Sci. 58, 103-116.

(59) Exton, J. H. and Park, C. R. (1967) J. Biol. Chem. 242, 2622-2636.

(60) Tolbert, M. E. M., Butcher, F. R., and Fain, J. N. (1973) J. Biol. Chem. 248, 5686-5692.

(61) Tolbert, M. E. M. and Fain, J. N. (1974) J. Biol. Chem. 249, 1162-1166.

(62) Haft, D. E. and Miller, L. L. (1958) Am. J. Physiol. 192, 33-42.

(63) Haft, D. E. (1967) Am. J. Physiol. 213, 219-230.

(64) Sols, A., Salas, M. and Viñuela, E. (1964) Adv. Enz. Regul. 2, 177-188.

(65) Sharma, C., Manjeshwar, R., and Weinhouse, S. (1964) Adv. Enz. Regul. 2, 189-200.

(66) McCraw, E. F., Peterson, M. J., Yarnell, G. and Ashmore, J. (1968) Adv. Enz. Regul. 6, 57-65.

(67) Cook, G. A., Sullivan, A. C. and Ontko, J. A. (1977) Arch. Biochem. Biophys. 179, 310-321.

(68) Marshall, R. D. (1972) Ann. Rev. Biochem. 41, 673-702.

(69) Brunengraber, E. G. and Javaid, J. I. (1975) Biochim. Biophys. Acta 404, 67-73.

(70) Javaid, J. I., Hof, H. and Brunngraber, E. G. (1975) Biochim. Biophys. Acta 404, 74-82.

(71) Margolis, R. K., Margolis, R. U., Preti, C. and Lai, D. (1975) Biochem. 14, 4797-4804.

(72) Ghosh, A. K., Mukherji, B. and Sloviter, H. A. (1972) J. Neurochem. 19, 1279-1285.

(73) Sols, A. and Crane, R. K. (1954) J. Biol. Chem. 210, 581-597.

(74) Viñuela, E., Salas, M. and Sols, A. (1963) J. Biol. Chem. 238, PC 1175-1177.

(75) Slein, M. W. (1950) J. Biol. Chem. 186, 753-761.

(76) Bruns, F. H., Noltmann, E. and Willemsen, A. (1958) Biochem. Z. 330, 411-420.

(77) Slein, M. W. (1955) in "Methods in Enzymology" (Colowick & Kaplan, ed.) Vol. I p. 299-304, Academic Press, New York.

(78) Gracy, R. W. and Noltmann, E. A. (1968) J. Biol. Chem. 243, 3161-3168.

(79) Gracy, R. W. and Noltmann, E. A. (1968) J. Biol. Chem., 243, 4109-4116.

(80) Gracy, R. W. and Noltmann, E. A. (1968) J. Biol. Chem. 243, 5410-5419.

(81) Noltmann, E. A. (1972) in "The Enzymes" (Boyer, P. D., ed.) Vol. VI, p. 271-307, Academic Press, New York.

(82) Murata, T. (1975) Plant & Cell Physio. 16, 953-961.

(83) Murata, T. (1975) Plant & Cell Physio. 16, 963-970.

(84) Manochiopinig, S. (1977) M.S. Thesis, Oklahoma State University.

(85) Munch-Petersen, A. (1955) Arch. Biochem. Biophys. 55, 592-593.

(86) Preiss, J. and Wood, E. (1964) J. Biol. Chem. 239, 3119-3126.

(87) Lin, T. Y. and Hassid, W. Z. (1966) J. Biol. Chem. 241, 5284-5297.

(88) Lowry, O. H. and Passonneau, J. V. (1969) J. Biol. Chem. 244, 910-916.

(89) Asikin, N. and Koeppe, R. E. (1979) Biochem. Biophys. Res. Comm. 89, 279-285.

(90) Wagle, S. R. and Ingebretsen, W. R., Jr. (1974) Proc. Soc. Exptl. Biol. Med. 147, 581-584.

(91) Seglen, P. O. (1975) in "Methods in Cell Biology" (D. M. Prescott, ed.) Vol. XIII, p. 72-83, Academic Press, New York.

(92) Walker, P. R. (1977) Biochim. Biophys. Acta 496, 255-263.

(93) Noguchi, T., Aramaki, Y., Kameji, T., Hayashi, S. (1979) J. Biochem. 85, 953-959.

(94) Paul, J. (1970) in "Cell and Tissue Culture", 4th ed., Livingston, Edinburgh.

(95) Williamson, J. R. and Corkey, B. E. (1969) in "Methods in Enzymology" (Lowenstein, ed.) Vol. XIII, p. 434-513. Academic Press, New York.

(96) Hulbert, R. B. (1957) in "Methods in Enzymology" (Colowick & Kaplan, ed.) Vol. III, p. 793, Academic Press, New York.

(97) Smith, M. and Khorana, H. G. (1963) in "Methods in Enzymology (Colowick & Kaplan, ed.) Vol. VI, p. 659, Academic Press, New York.

(98) Mayer, D. H., Williamson, J. R. and Legallais, V. (1969) Chem. Instrument. 1, 383-389.

(99) Lowry, O. H. and Passonneau, J. V. (1972) "A Flexible System of Enzymatic Analysis", p. 146-218, Academic Press, New York.

(100) Asikin, N. (1978) Ph.D. Dissertation, Oklahoma State University.

(101) Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. and Schulz, D. W. (1964) J. Biol. Chem. 239 18-30.

(102) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.

(103) Dickson, A. J. and Langslow, D. R. (1975) Bioch. Soc. Trans. 3, 1034-1037.

(104) Howard, R. B., Lee, J. C., and Pesch, L. A. (1973) J. Cell Biol. 57, 642-658.

(105) Seglen, P. O. (1972) Exp. Cell Res. 74, 450-454.

(106) Gallop, P. M., Selfter, S. and Meilman, E. (1957) J. Biol. Chem. 227, 891-906.

(107) Seglen, P. O. (1972) Biochim. Biophys. Acta 264, 398-410.

(108) Berg, T., Bowman, D. and Seglen, P. O. (1972) Exp. Cell Res. 72, 571-574.

(109) Good, N. E., Winget, D. G., Winter, W. and Connolly, T. N. (1966) Biochem. 5, 467-477.

(110) Cappuzzi, D. M., Rothman, V., and Margolis, S. (1971) Biochem. Biophys. Res. Comm. 45, 421-429.

(111) Cappuzzi, D. M., Rothman, V. and Margolis, S. (1974) J. Biol. Chem. 249, 1286-1294.

(112) Omistorff, B., Bondesen, S. and Grunnet, N. (1973) Biochim. Biophys. Acta 320, 503-516.

(113) Hems, R., Lund, P. and Krebs, H. A. (1975) Biochem. J. 150, 47-50.

(114) Sainsbury, G. M., Stubbs, M., Hems, R. and Krebs, H. A. (1979) Biochem. J. 180, 685-688.

(115) Geelen, M. J. H. and Gibson, D. M. (1976) in Ref. 37, p. 219-232.

(116) Sharma, C., Manjeshwar, R. and Weinhouse, S. (1963) J. Biol. Biochem. 238, 3840-3845.

(117) Start, C., Newsholme, E. A. (1968) Biochem. J. 107, 411-415.

(118) Hornbrook, K. R., Burch, H. B. and Lowry, O. H. (1965) Biochem. Biophys. Res. Comm. 18, 206-211.

(119) Sols, A. and Crane, R. K. (1954) J. Biol. Chem. 210, 581-597.

(120) Walker, D. G. and Parry, M. J. (1966) in "Methods in Enzymology" (Wood, ed.) Vol. IX, p. 381-392, Academic Press, New York.

(121) Crane, R. K. and Sols, A. (1955) in "Methods in Enzymology" (Colowick & Kaplan, ed.) Vol. I, p. 277-286, Academic Press, New York.

(122) Scrulton, M. C. and Utter, M. F. (1968) Ann. Rev. Biochem. 37, 249-302.

(123) Abraham, S., Borreback, B. and Chaikoff, I. L. (1964) J. Nutrition 83, 273-288.

(124) Newsholme, E. A. and Start, C. (1973) "Regulation in Metabolism" Wiley, London, p. 261-267.

(125) Dipietro, D. L. and Weinhouse, S. (1960) J. Biol. Chem. 235, 2542-2545.

(126) Walker, D. G. and Rao, S. (1964) Biochem. J. 90, 360-368.

APPENDIX

A SELECTED BIBLIOGRAPHY OF RELATED WORKS

ON ISOLATED HEPATOCYTES

Baur, H., and Heldt, H. W. (1977) Eur. J. Biochem. 74, 397-403.

Beynen, A. C., and Geelen, M. J. H. (1981) Indian J. Exp. Biol. 19, 46-48.

Billings, R. E., Ashmore, J., Wagle, S. R., and Mcmahon, R. E. (1975) Fed. Proc. 34, 727.

Chan, T. M., and Exton, J. H. (1976) Anal. Biochem. 71, 96-105.

Chan, T. M., Blackmore, P. F., Steiner, K. E., and Exton, J. H. (1979) J. Biol. Chem. 254, 2428-2433.

Chan, T. M., Steiner, K. E., and Exton, J. H. (1979) J. Biol. Chem. 254, 11374-11378.

Clark, D. G., Filsell, O. H., and Topping, D. L. (1979) Biochem. J. 184, 501-508.

Clark, R. L., and Hansen, R. J. (1980) Biochem. J. 190, 615-619.

Crabb, D. W., Mapes, J. P., Boersma, R. W., and Harris, R. A. (1976) Arch. Biochem. Biophys. 173, 658-665.

Craik, D. J., and Elliott, R. F. K. (1980) Biochem. J. 192, 373-375.

Erickson, R. R., and Holtzman, J. L. (1976) Biochem. Pharmacol. 25, 1501-1506.

Fix, J. A., and Moore, W. V. (1981) Endocrinology 108, 239-246.

Freude, K. A., Sandler, L. S., and Ziev, F. J. (1981) Am. J. Physiol. 240, E226-E232.

Genevieve, K., Gravier, O., Roberfroid, M., and Mercier, M. (1980) Biochim. Biophys. Acta 632, 619-629.

Hayes, J. S., and Brendel, K. (1976) Biochem. Pharmacol. 25, 1495-1500.

Henderson, P. T., and Dewaide, J. H. (1969) Biochem. Pharmacol. 18, 2087-2094.

Hensgens, H. E. S. J., Meijer, A. J., Gimpel, J. A., and Tager, T. M. (1978) Biochem. J. 170, 699-708.

Holtzman, J. L., Rothman, V., and Margolis, S. (1972) Biochem. Pharmacol. 21, 581-584.

Hopf, U., Meyer Zum Bueschenfelde, K-H., and Freudenberg, J. (1973) Verh. Dtsch. Ges. Inn. Med. 79, 663-666.

Hue, L., Feliu, J. E., and Hers, H. G. (1978) Biochem. J. 176, 791-798.

Hutson, N. J., Brumley, F. T., Assimacopoulos, F. D., Harper, S. C., and Exton, J. H. (1976) J. Biol. Chem. 251, 5200-5208.

Inaba, T., Umeda, T., Mahon, W. A., Ho, J., and Jeejeebhoy, K. N. (1975) Life Sci. 16, 1227-1232.

Kirk, C. J., Mitchell, R. H., and Hems, D. A. (1981) Biochem. J. 194, 155-165.

Kneer, N. M., Bosch, A. L., Clark, M. G., and Lardy, H. A. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4523-4527.

Krebs, H. A., Hems, R., and Tyler, B. (1976) Biochem. J. 158, 341-353.

Longenecker, J. P., and Williams, J. F. (1980) Biochem. J. 188, 847-858.

Longenecker, J. P., and Williams, J. F. (1980) Biochem. J. 188, 859-866.

Lund, P., Cornell, N. W., and Krebs, H. A. (1975) Biochem. J. 152, 593-599.

Lund, H., Borreback, B., and Bremer, J. (1980) Biochim. Biophys. Acta 620, 364-371.

Marchand, J. C., Lavoinne, A., Giro, M., and Matray, F. (1979) Biochimie 61, 11-12.

McLimans, W. F., Mount, D. T., Bogitch, S., Crouse, E. J., Harris, G., and Moore, G. E. (1966) Ann. N. Y. Acad. Sci. 139, 190-213.

Muller, P., Singh, A., Orci, L., and Jeanrenand, B. (1976) Biochim. Biophys. Acta 428, 480-494.

Rappaport, C., and Howze, G. B. (1966) Proc. Soc. Exp. Biol. Med. 121, 1010-1015.

Rognstad, R., and Katz, J. (1979) J. Biol. Chem. 254, 11969-11972.

Wagle, S. R., Ingebretsen, W. R., Jr., and Sampson, L. (1973) Diabetes 22, 330.

Wagle, S. R., and Ingebretsen, W. R., Jr. (1974) Diabetes 24, 384.

Wagle, S. R. (1975) Life Sci. 17, 827-835.

Wagle, S. R. (1975) Biochem. Biophys. Res. Comm. 67, 1019-1027.

Wagle, S. R. (1976) Acta Diabetol. Lat. 13, 186-201.

Walajtys-Rode, E., Coll, K. E., Williamson, J. R. (1979) J. Biol. Chem. 254, 11521-11529.

Wirthensohn, G., Brocks, D. G., and Guder, W. G. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 985-994.

Witters, L. A., Alberico, L., and Avruch, J. (1976) Biochem. Biophys. Res. Comm. 69, 997-1003.

Schudt, C. (1980) Biochim. Biophys. Acta 629, 499-509.

Sprandel, U., Wolfram, G., and Scholz, R. (1976) Res. Exp. Med. 168, 157-164.

Strickland, W. G., Blackmore, P. F., and Exton, J. H. (1980) Diabetes 29, 617-622.

Young, P. S., and Aisen, P. (1980) Biochim. Biophys. Acta 633, 145-153.

VITA

Sudarat Manochiopinig

Candidate for the Degree of

Doctor of Philosophy

Thesis: MANNOSE-6-PHOSPHATE CONCENTRATIONS IN HEPATOCYTES AND PERFUSED RAT LIVERS

Major Field: Biochemistry

Bibliographical:

Personal Data: Born in Bangkok, Thailand, September 18, 1948, the daughter of Mr. Yeo Kiang Lo and Mrs. Sin Eng Erng.

Education: Graduated from Triam Udom Suksa High School, Bangkok, Thailand in March, 1968; received the Bachelor of Science in Medical Technology degree (2nd honor) from Mahidol University in March, 1972; received Master of Science degree in Biochemistry from Oklahoma State University in December, 1977; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in December, 1981.

Professional Experience: Employed by Royal Thai Governments as a research fellow at Mahidol University, Division of Hematology, Department of Medicine, Faculty of Medicine and Siriraj Hospital from 1972 to present; served as research assistant at Oklahoma State University from 1978 to 1981.

Professional Organizations: Associate Member of the Society of Sigma Xi.