A STUDY OF MANNOSE METABOLITES IN RAT TISSUES

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LIST OF SYMBOLS AND ABBREVIATIONS

PCA	- perchloric acid
TEA	- triethanolamine
EtOH	- ethanol
HA	- hydroxyapatite
Glc1P	- glucose-1-phosphate
Glc6P	- glucose-6-phosphate
Fru6P	- fructose-6-phosphate
Man1P	- mannose-1-phosphate
Man6P	- mannose-6-phosphate
Glc(1,6)P_2	- glucose-1,6-bisphosphate
Man(1,6)P_2	- mannose-1,6-bisphosphate
6PGlcA	- 6-phosphogluconic acid
UDP-Glc	- uridine diphosphoglucose
GDP-Fuc	- guanosine diphosphofucose
GDP-Man	- guanosine diphosphomannose
GDP-ManUA	- guanosine diphosphomannuronic acid
HK	- hexokinase
PGI	- phosphoglucose-isomerase
PMI	- phosphomannose-isomerase
PMM	- phosphomannomutase
PGM	- phosphoglucomutase
G6PDH	- glucose-6-phosphate dehydrogenase

CHAPTER I

INTRODUCTION

This study was carried out in an attempt to determine the concentration of mannose metabolites in rat tissues. The interest of this laboratory in the study of mannose metabolism was first stimulated by the report, in 1972, of Sloviter et al. (1) that mannose could replace glucose as an energy substrate for perfused rat brain. After a fifty-minute perfusion with either glucose or mannose, at a rate of 4 mg/min (0.022 mmol/min) the level of mannose-6-phosphate (Man6P) was found to be 320 and 475 nmol/g of tissue, respectively. The concentration of Man6P in the cerebral tissue of the normal rat was found to be similar to that of the isolated brain perfused with glucose. A year later (2) the isolation of Man6P from rat brain by ion-exchange chromatography was described.

In the study above it was demonstrated that there was no substantial change in the pattern of metabolism of the brain perfused with mannose compared to that perfused with glucose. Mannose maintains the energy reserves (adenine nucleotides, creatine phosphate) and oxidative status (lactate/pyruvate and α-glycerophosphate/dihydroxyacetone-phosphate ratio) of the perfused tissue, and does not alter the levels of cerebral free amino acids, which appear to function as substrate reservoirs.

An earlier study using the isolated, perfused rat brain showed

that the electrical and metabolic activity of the brain was maintained just as well with mannose as metabolic substrate as with glucose (3). Samples of the perfusion fluid contained low levels of glucose (less than 20 mg/100 ml), indicating no appreciable conversion of mannose to glucose.

Mannose does not normally constitute an important part of an animal diet; however, the similarity in structure makes it conceivable that the body could utilize mannose in place of glucose. As early as 1922 (4) mannose injection was shown to relieve the symptoms of hypoglycemia resulting from hepatectomy, and to restore to normal the electrical activity of brains of rabbits rendered hypoglycemic by hepatectomy or by insulin injection (5). At that time it was thought that mannose was first converted to glucose by tissues other than the brain.

Chain et al. (6) observed that rat cerebral cortical slices metabolized (U-^{14}C)mannose essentially like glucose based on oxygen utilization, CO_2 production, and incorporation of radioactivity into lactate, aspartate, glutamate and γ-aminobutyric acid.

Studies on the metabolism of mannose have been done on tissues other than the brain. The utilization of mannose by adipose tissue and liver slices from normal and alloxan-diabetic rats was shown to be identical to that of glucose; the metabolism of both sugars was suppressed in the diabetic tissues and stimulated by insulin (7). A similar finding was reported on the metabolism of both sugars by kidney slices of normal and diabetic rats, diabetic tissues showed a decreased incorporation of radioactivity into CO_2, fatty acids and glycogen (8).

McNamara et al. (9) studied the transport and metabolism of (1-^{14}C)mannose in rat renal cortical slices. The uptake process appear-

ed to be saturable and oxygen dependent and to be inhibited by glucose, galactose and phlorizin, indicating an interaction with the glucose transport system. $^{14}CO_2$ was produced at a rate comparable to that from glucose. Radioactive compounds accumulated in the tissue, about 50% of which were in the form of phosphorylated intermediates (precipitable by $Ba(OH)_2$ and $ZnSO_4$). The remaining neutral fraction, analyzed by gas-liquid chromatography, consisted of mannose (83%), glucose (5%) and an unidentified compound which behaves like an alditol (9%). Assuming a direct conversion to glucose, the specific activity of glucose indicated that 28% of it was derived from mannose. An earlier study by Krebs and Lund (10) using slices of rat renal cortex incubated with 10 mM mannose showed that glucose was formed at a rate of 50 µmol/h per gram of tissue.

It has also been observed, in slices of guinea pig skin, that respiration can be supported by mannose to the same extent as by glucose. Man6P was detected on paper radioautographs of normal skin maintained on glucose. Skin maintained on mannose produced chromatograms indistinguishable from those of skin maintained on glucose (11).

Mannose has a widespread occurrence in the plant and animal kingdom and it is a component of various cellular constituents of functional importance to organisms. The following is a list of some of the compounds shown to contain mannose:

1) Cell-wall mannans of plants. The mannan of M.lysodeikticus was shown to be a lipopolysaccharide (12) and mannans in the yeast cell-wall provide structural functions as well as attachment sites for extracellular enzymes such as invertase and alkaline phosphatase (13).

2) Glycolipids containing mannose, inositol and phosphoryl-ceramide have been described in yeast (14) and are related to the phytoglycolipids from various plant seeds (15). The glycolipid of the fungus U. maydis (16) contains erythritol, whereas those of mycobacteria contain diglyceride and have antigenic properties (17,18,19).

3) A lipopolysaccharide of M. phlei containing 3-O-methyl mannose was shown to stimulate fatty acid synthetase by decreasing the Km for acetyl-CoA and malonyl-CoA (20). The O-antigen of salmonellae consists of a repeating unit of the trisaccharide mannosylrhamnosylgalactose (21).

4) Membrane glycoproteins serve an important role in cell-cell interaction, differentiation and growth, and as antigenic determinants (22).

5) Secretory glycoproteins, including egg-albumin (23), lactoferrin (24) and the plasma glycoproteins orosomucoid, transferrin, prothrombin and fibrinogen (25). Also containing mannose are the immunoglobulins (26), luteinizing and follicle-stimulating hormones and thyroglobulin (25), the visual pigment rhodopsin (27) and the enzymes ceruloplasmin (superoxide dismutase), hexokinase (22) and pancreatic ribonucleases and lipases (28,29,30).

The carbohydrate moieties of several glycoproteins appear to have only a structural and not a functional significance. As an example, prothrombin, fibrinogen, transferrin and γ-globulin have biological activities which appear to be independent of the bound carbohydrate. Ribonuclease A and B have identical amino acid sequences and differ only by a carbohydrate unit (in RNase B) but have identical substrate speci-

ficity and enzymatic activity (25).

A comparison between extracellular and intracellular proteins indicated that the carbohydrate unit of extracellular proteins acts as a label which destines the proteins for secretion. It would seem logical that the carbohydrate unit, by interaction with an appropriate receptor or carrier, would act as a chemical passport for exit through the cellular membrane (25).

Glycoproteins could be classified into 3 types (31):

1) Serum or plasma glycoproteins where each glycan unit is linked through an N-glycosidic bond between an N-acetylglucosamine (GlcNAc) residue at the reducing end of the unit and an asparagine (Asn) residue in the protein core. This type of glycoprotein contains mannose at the core region. All plasma proteins, except albumin, appear to be glycoproteins of this type.

2) Mucin type glycoproteins usually have a di- or penta-saccharide unit linked to the threonine or serine residues of a protein chain through an O-β-glycosidic bond with N-acetylgalactosamine (GalNAc). These glycoproteins do not contain mannose or GlcNAc; most plasma membrane glycoproteins are of this type. (Yeast cell-wall mannans also have this type of linkage, with GlcNAc).

3) Mucopolysaccharides or proteoglycans which are characterized by the presence of uronic acids and absence of sialic acids.

In recent years active research in many laboratories has been conducted to elucidate the mechanism and regulation of the biosynthesis of polysaccharides and glycoproteins in bacteria, yeast, plants and animals. The systems studied include bacteria (for review see 21), yeast (32,33), mung bean (34), cotton-boll (35), aorta (36), rat and calf

brain (37,38), rat and bovine thyroid (39,40), pig liver (41), bovine retina (42), chicken oviduct (43) and human lymphocytes (44).

What might now be considered the classical pathway for the biosynthesis of complex carbohydrates involves the sequential transfer of individual monosaccharides from their nucleotide derivatives to acceptor molecules in a process mediated by specific glycosyl-transferases. Knowledge of the overall biosynthetic pathway is still incomplete; not all the steps have been rigorously documented and none of the enzymes involved have been isolated and purified.

It is evident that lipid bound sugars are intermediates in the biosynthetic process. The lipid moieties of the glycolipid intermediates in bacterial and plant systems have been shown to be polyisoprenoids of 10 - 12 isoprene units (bactoprenol, betulaprenol), whereas the dolichols, a class of polyisoprenoids containing 17 - 22 isoprene units with a saturated terminal (alcohol end), are lipid carriers found in mammalian systems and yeast.

The mannose containing glycoproteins of animals appear to have a common "core" region in their oligosaccharide chain, having the general structure:

$$(Man)_x\text{-}Man(1\text{-}4)\text{-}GlcNAc(1\text{-}4)\text{-}GlcNAc\text{-}\ Asn(polypeptide)$$

This core unit may or may not have an attached side chain of GlcNAc, galactose and sialic acid (or fucose).

Cytological and radioautographic evidence indicate that the glycoprotein being synthetized flows from the rough and smooth endoplasmic reticulum through the Golgi apparatus and secretory vesicles to the

plasma membrane and extracellular space or to intracellular structures such as lysosomes. The core unit is first synthetized attached to the lipid carrier as shown in Figure 1, and then transferred in toto to the growing peptide chain in the rough endoplasmic reticulum (43). The addition of terminal sugars occurs in the smooth endoplasmic reticulum and the Golgi apparatus (39,45), where the nucleotide sugar derivatives (UDP-GlcNAc, UDP-Gal, UDP-Glc and GDP-Fuc) appear to be the donor molecules. Inversion of anomeric configuration usually occurs at each glycosylation such that the configuration of the newly attached sugar is opposite to that in the donor.

The presence of the tripeptide sequence Asn-X-Ser(Thr) appears to be necessary but not sufficient for the protein to serve as acceptor, suggesting that the unfolding of the polypeptide chain is required to expose the appropriate Asn site and that the polypeptide folding may play an important role in the regulation of protein glycosylation (46). The glycosyltransferases in the membranes of the smooth endoplasmic reticulum and Golgi apparatus exhibit a high degree of specificity for sugar donor and for the oligosaccharide structure of the glycoprotein acceptor (47), thus adding another control mechanism.

Of interest is the possible involvement of vitamin A (retinol) as a lipid carrier in glycoprotein biosynthesis. It was well known that epithelial cells generally lose their mucus secreting ability in vitamin A deficiency and may be keratinized (cornea) or not (intestine). The goblet cells of vitamin A deficient rats showed an impaired synthesis of fucose containing glycoproteins (48). Retinyl-phosphate was shown to function as an acceptor of mannose from GDP-Man in an in vitro system from rat liver membranes; the incorporation of labelled mannose into

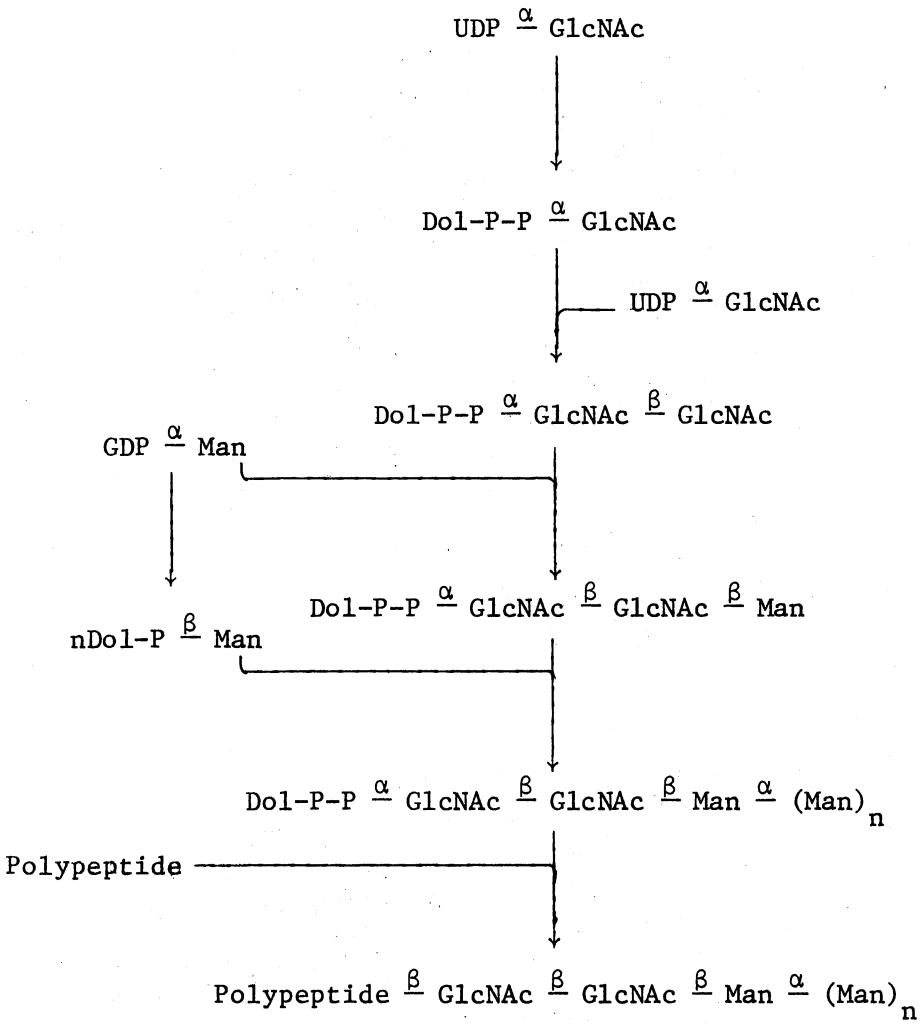


Figure 1. Synthesis of "Core" Oligosaccharide.

glycolipid and glycoprotein decreased by 60 - 90% in mild and severe vitamin A deficiency compared to that of vitamin A fed hamsters (49). It was also observed that mannosyl-retinyl phosphate represents 5 - 10% of the total labelled mannolipid synthetized by normal rat liver membranes from labelled GDP-Man, the remainder being dolichol-P-Man (50).

Brunngraber et al. (51) described mannose-rich glycoproteins from rat brain, over 50% of which are localized in the synaptosomal and microsomal fractions. Margolis et al. (52) isolated mannose-rich glycopeptides from Pronase digested rat brain nuclei and also studied the developmental changes in rat brain glycoprotein. Between 1 - 30 days postnatal a larger increase (2 - 3 times) was observed in the core sugars than in those of the side chain, suggesting a preferential synthesis of glycoproteins containing primarily "core" units (37).

We owe much of our understanding of polysaccharide and glycoprotein biosynthesis to L.F.Leloir, who first discovered the sugar nucleotides which were later shown to function as glycosyl donors. UDP-glucose was discovered (53) in yeast in 1949 and GDP-mannose in 1953 (54). GDP-Man was later detected in several animal sources: bovine aorta, 8 nmol/g (55), hen egg-white, 0.18 µmol/ml, representing 90% of the total nucleotide (56), hen oviduct (isthmus), 11 nmol/g, and uterus, 0.1 nmol/g (57), rabbit uterus, 70 nmol/g (58) and human red-blood-cells, 20 - 60 nmol/ml (59). More GDP-Man was found in colostrum than in milk; the colostrum of cow, human and pig, respectively, contains 1.9, 0.11 and 34 µmol/100 ml (60,61).

GDP-Man formation is catalyzed by the enzyme GDP-Man pyrophosphorylase. The enzyme was first described in yeast (62) and later in bacteria and algae (63,64). Hansen el al. (65,66) found that calf liver

and bovine and rat mammary gland were good sources of the enzyme; no activity was detected in hen oviduct. The enzyme is nonspecific for the nucleotide or sugar moiety; in decreasing order of activity, GDP-, IDP-, and ADP-hexoses are substrates with either glucose or mannose as the sugar moiety. The reaction catalyzed by the enzyme is reversible, thus:

$$GTP + Man1P \rightleftharpoons GDP\text{-}Man + PPi$$

Fucose in glycoproteins is incorporated from GDP-fucose, which is formed from GDP-Man by the enzyme GDP-mannose oxidoreductase. The reaction was first described in A.aerogenes and requires NADPH (67). GDP-fucose was found in cow (30 nmol/ml) but not in human colostrum (60). L-fucose was found in human milk (6.7 μmol/ml) and (6-^{14}C)fucose was recovered in human milk after intravenous administration of (6-^{14}C) glucose (68). Of interest also was the oxidation of GDP-Man to GDP-mannuronic acid in the soil bacteria A. viscosus (69). This reaction is catalyzed by GDP-Man dehydrogenase and requires NAD.

From the discussion above we see that glucose can be converted to Man6P and the mannose moiety of glycoproteins. Using calf thyroid slices incubated with labelled glucose, Spiro and Spiro (70) demonstrated an early incorporation of radioactivity into the galactose moiety of thyroglobulin. Mannose was labelled after one hour and sialic acid after two hours.

On the other hand, available mannose is incorporated into glycoproteins and enters the glycolytic pathway to serve as a source of energy. How is this interconversion carried out? Sols and Crane (71) reported that mannose is phosphorylated by hexokinase to Man6P, and

Chalkoff et al. (72) reported mannokinase activity in rat tissues.

The enzyme phosphomannose isomerase (PMI), which is distinct from phosphoglucose isomerase (PGI) and interconverts fructose-6-phosphate (Fru6P) and Man6P, was first described in rabbit muscle (73). Noltmann et al. (74) demonstrated the presence of PMI in the tissues of pig and rat, and separated the yeast PMI from PGI (75). The yeast enzyme was further characterized by Gracy and Noltmann (76) and was shown to be a zinc metalloenzyme of molecular weight 45 000.

A question concerning the pathway from glucose to GDP-mannose is how is Man1P formed? Thus far Man1P has only been reported to be present in the mold P. chrysogenum, about 90 mg being recovered, by calcium acetate and ethanol fractionation, from 1 kg of mycelium (77). In 1959, Glaser (78) reported the presence of a distinct phosphomannomutase (PMM) in yeast, catalyzing the interconversion of Man6P and Man1P. The K_m's for glucose-1,6-bisphosphate (Glc(1,6)P_2) and mannose-1,6-bisphosphate (Man(1,6)P_2) are 0.16 and 0.12 mM, respectively, which are higher than the corresponding K_m's of muscle or yeast phosphoglucomutase (PGM). The enzyme has not been reported in animal tissues even though it was assumed to function in the synthesis of fucosyllactose in milk (79).

It is possible that the interconversion of Man6P and Man1P is catalyzed by PGM, however the reaction is much slower than that for Glc6P and Glc1P. The yeast enzyme has a K_m and V_m for Man1P of 245 µM and 19.4 µmol/mg per min, respectively. The corresponding values for Man6P are 500 and 0.2, and for Glc1P, 8 and 328.

Another branch of the metabolism of mannose is the formation of L-neoinositol-1-phosphate (nIns1P) from Man6P. This reaction is catalyzed by hexose-6-phosphate inositol-1-phosphate cyclase, the same

enzyme for the formation of L-myoinositol-1-phosphate from Glc6P (81). Neo-inositol has been identified from calf brain by gas-liquid chromatography mass spectrometry of its trimethylsilyl- and acetate esters. The compound is present in micromolar amounts in rat tissues except liver, the highest concentration (14 μM) being in the testes (81).

The metabolism of mannose is depicted in Figure 2. Man(1,6)P_2 which is supposed to be the intermediate/coenzyme in the mutase reaction (PGM,PMM) was found in the erythrocytes of humans and various mammals at a concentration of 0.1 - 0.2 μmol (phosphate) per ml (82). This metabolite was shown to be an effective activator of red cell pyruvate kinase in vitro (83), and considering its high concentration it may well function as an important metabolic regulator.

Rose (84) has recently reported that mouse brain contains an enzyme, glucose-1,6-bisphosphate synthase, catalyzing the reaction:

$$\text{glycerate-1,3-P}_2 + \text{Glc1P} \longrightarrow \text{glycerate-3-P} + \text{Glc(1,6)P}_2$$

Glc6P and Man1P were good alternate acceptors suggesting that this enzyme might function in the synthesis of Man(1,6)P_2. However, the enzyme was later shown to be inhibited by physiological concentrations of fructose-1,6-bisphosphate (Fru(1,6)P_2), Glc(1,6)P_2, phosphate and citrate (85).

In this study I determined the concentration of the metabolites of mannose, Man6P and Man1P, in brain and other tissues of rat. The effects of animal age and of the injection of sugar precursors, glucose, fructose and mannose on these concentrations were studied. Of the tissues studied,

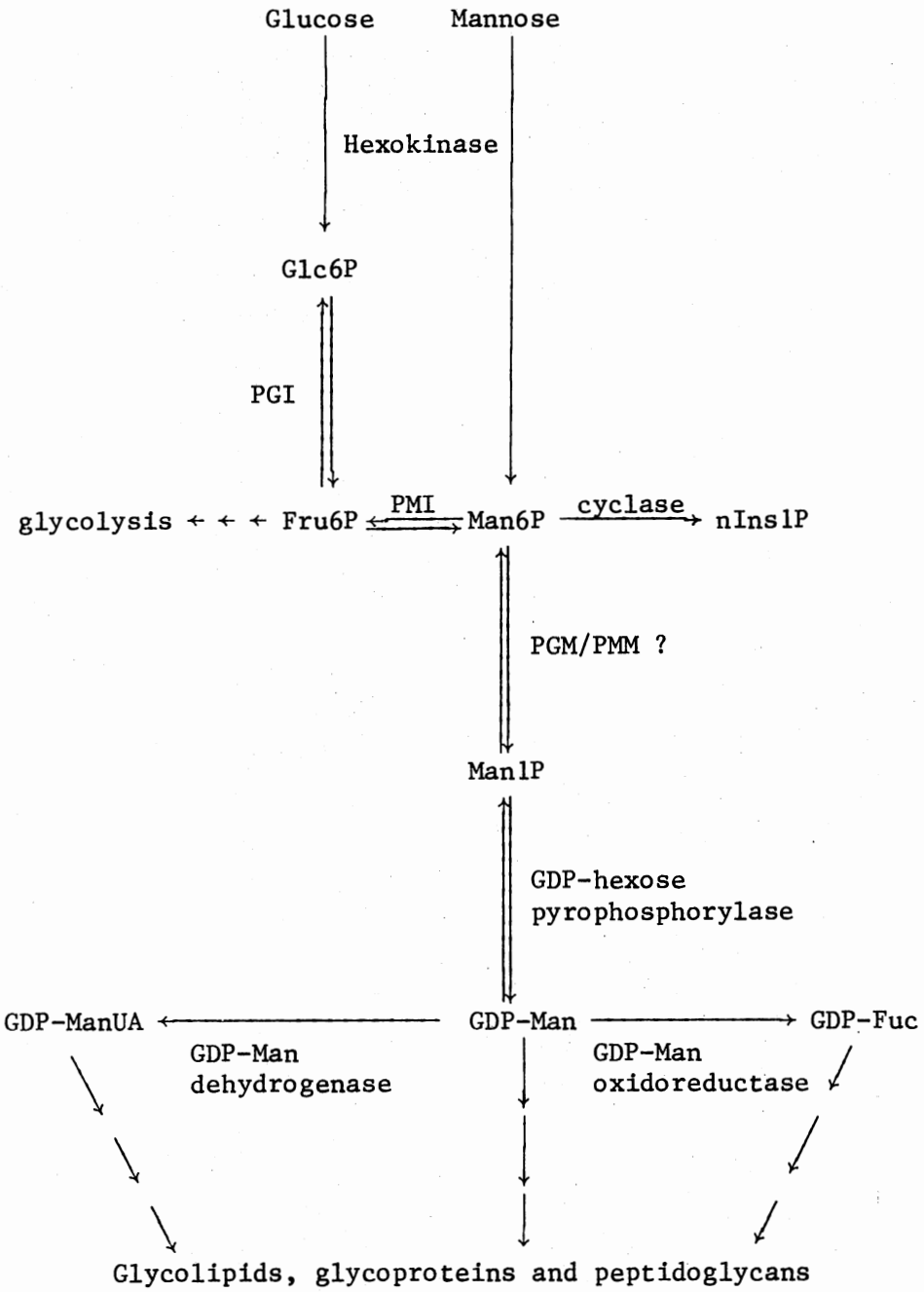


Figure 2. Pathway of Mannose Metabolism in Plants and Animals.

the highest concentration of Man6P and Man1P was found in the liver, about 100 and 15 nmol/g, respectively. Mannose loading resulted in a variable but significant increase of liver Man6P and Man1P, with an apparent decrease in ATP.

CHAPTER II

MATERIALS AND METHODS

Materials

Glucose, fructose and perchloric acid were from J. T. Baker. ATP, NADP, GDP-Man, hexose-phosphates, mannose and triethanolamine (TEA) base were from Sigma. PMI was from Boehringer, the other enzymes were from either Boehringer or Sigma. Hydroxyapatite (Biogel-HTP) and the ion-exchangers AG1-X4, 100-200 mesh and AG50-X4, 50-100 mesh were from Bio-Rad. Charcoal (Norit A) was from Matheson. Polyethyleneimine (PEI) cellulose thin layer plates were from Analtech.

Deionized distilled water was used for the preparation of reagents and solutions. All solutions, samples and enzymes used in fluorometric assays were filtered through 0.45 μ Millipore filter.

Absorbance measurements were carried out on a Hitachi-Perkin-Elmer model 124 spectrophotometer equipped with a Coleman 165 recorder. A Johnson Research Foundation Metabolite Fluorometer was used for fluorescence measurements. The fluorometer is supplied with a low pressure mercury lamp and an excitation filter with maximum transmission at 355 nm and 53 nm half-width, thereby allowing the 334 and 365 nm mercury lines to pass through. Filters were used on the emission side to exclude wavelengths below 420 nm and above 500 nm (86).

Chromatographic eluates were monitored at 254 nm using an ISCO UA-2 analyzer; fractions were collected with an RSCo fraction collector.

Methods

Preparation of Tissue Extracts

In the earlier part of this study, tissues were obtained from rats frozen whole in liquid nitrogen. As this method did not permit quick freezing of the tissues of interest and gave results that did not reflect in vivo conditions, the method was later abandoned except for smaller animals (about 60 g or less).

Female Holtzmann rats were used throughout, except for the young ones and, of course, for testes. They were brought to the laboratory and acclimated to handling for at least two days before being killed; they were allowed free access to food and water.

The rats were usually killed around 10 - 12 A.M. Each was anesthetized first by placing in a jar containing Kimwipes saturated with ether, the lid (a used TLC plate) being opened just enough so the animal could not escape. As soon as the rat became unconscious, it was placed on the lab bench, with the head in a beaker containing Kimwipe saturated with ether.

The skin above the forehead was removed and the abdominal cavity was exposed by making a midline incision towards the diaphgragm and laterally along the costal margin. The rat was then immersed in the refrigerant (liquid nitrogen) and the desired tissues were chiseled out from the frozen carcass. When the brain was not required the tissues desired were excised and pressed with a porcelain pestle (precooled with liquid nitrogen) in a mortar filled with refrigerant, a flattened tissue was obtained within 5 sec after excision. Blood was obtained from a cut of the tail and collected in a tube, cooled in an ice bath,

containing the appropriate amount of potassium oxalate as antocoagulant. A sample of whole blood was quickly deproteinized for assay.

Glucose, fructose or mannose was injected intraperitoneally, under ether anesthesia, at a dose of 20 mmol/kg body weight given as a 25% solution. The animal was allowed to recover and after an appropriate interval was reanesthetized and killed; a successful injection was indicated by the accumulation of fluid in the abdominal cavity.

Fat and excess blood were removed from the tissues obtained by either of the procedures above without allowing them to thaw (periodic immersion into liquid nitrogen). They were put in a steel mortar surrounded by dry ice and pulverized. The tissue powder was transferred with a scoopula (precooled with liquid nitrogen) into a glass homogenizing tube cooled in dry ice. The weight of the powder was determined by the difference of the weight of the tube before and after the addition of the powder; weighing was done on a Torbal torsion balance or, more conveniently, with a Fisher electronic balance (thanks to Ms. Judy Hall for lending the balance).

When the recovery of a metabolite was determined, the tissue powder was transferred into two homogenizing vessels and, after weighing, a known amount of the solution of the metabolite (i.e. hexose-phosphate) was added to one of them. On a given day this was done only with one of the tissue samples; the recovery of the other samples was assumed to be identical.

The tissue powder was deproteinized by adding 4 to 6 volumes of a mixture of 8% perchloric acid-40% ethanol (PCA-EtOH) (87) and, after mixing well and standing in an ice bath, homogenized in the Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was trans-

ferred with a Pasteur pipet into a round-bottomed centrifuge tube. The residual homogenate was quantitatively transferred by the addition of 1 ml, 0.5 ml and 0.5 ml of PCA-EtOH, homogenizing each time before transferring to the centrifuge tube. This method gave a satisfactory recovery and there was no need to rehomogenize the protein pellet after centrifugation, as was later found to be verified in the literature (88).

The homogenate was centrifuged at 10 000 x g for 20 min in the SS-1 rotor of a Servall centrifuge. The supernate was transferred into another centrifuge tube and neutralized to a pH of about 6 by the slow addition of 3 M K_2CO_3 containing 0.5 M TEA base (87) while mixing with a magnetic stirrer. In later experiments where Man1P was to be determined, neutralization was done with 5 M KOH; the use of a buffer was a disadvantage here because of the requirement of alkaline and acid conditions for hydrolysis.

Since the extracts of liver and kidney were yellowish brown in color and highly fluorescent, charcoal was added at 10-20 mg/ml for decolorization (89). It was observed that charcoal should be added before neutralization was carried out, otherwise decolorization was not efficient. For nucleotide determination, an aliquot of the homogenate was used without decolorization. The charcoal used in this study was prewashed (100 g) successively with 2 l of 1 M HCl, 2 l of 1 M ammonia and water until neutral followed by drying for 18 h in an oven at 110^0C (90); the charcoal was then stored in a tightly stoppered bottle.

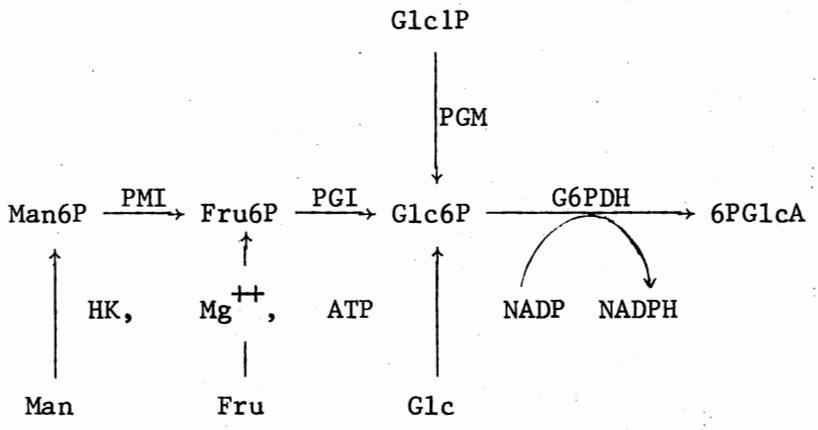
Potassium perchlorate was allowed to precipitate for 30 min in the cold followed by centrifugation at 10 000 x g for 30 min. The neutral-

ized extract, stored at 4^0C for 5 days, showed no change in hexose-6-phosphate concentration. ATP was usually determined on the same day, although Williamson mentioned that hexose mono- and di-phosphates and ATP were stable for at least 4 days (88). Aliquots for the determination of Man1P, which is stable in alkali, were immediately treated with KOH to 0.05 N and stored at 4^0C before further treatment. Assays were always completed within one week after the preparation of homogenates.

Assay of Metabolites

General. All assays were carried out in 0.1 N TEA buffer pH 7.6 at 30^0C. ATP and glucose, or fructose and mannose after injection of the sugars, were determined spectrophotometrically; the hexose-phosphates were determined on the fluorometer. In each case the metabolites were determined by an enzymic assay using a reaction coupled to glucose-6-phosphate dehydrogenase (G6PDH) and measuring the increase in absorbance or fluorescence of the NADPH being formed (87,91).

The reactions were as follows:



Assays were done in 2 ml final volume containing the indicated quantities of the following reagents as appropriate: $MgCl_2$, 3 mM; ATP, 500 μM; NADP, 400 μM; PMI (yeast), 1.5 U; HK (yeast), 1.5 U; PGI (yeast), 1 U; PGM (rabbit muscle), 1 U; G6PDH (yeast), 1 U; glucose, 1 mM.

Under the conditions described above the PMI reaction was completed in 15 to 20 min. After the Man6P portion of the assay was started by the addition of PMI, the cuvette was set aside in the incubation chamber of the fluorometer and a second assay was started. After the addition of PMI to the second cuvette, the first sample was returned to the assay chamber, care being taken to put it in the original alignment, and the fluorescence increase was read. In this way the total assay time was reduced substantially.

The size of the sample was chosen so that the total increase in absorbance was 0.4 or less to ensure a linearity in the response. For the same reason the total amount of metabolites being sequentially assayed was made less than 20 nmol/2 ml (fluorometric).

The amount of metabolite was calculated assuming a molar absorbance of NADPH of 6.22×10^6 $cm^{-1}M^{-1}$. The fluorometer was standardized by determining the fluorescence developed using a solution of glucose, Glc6P or NADPH of known absorbance or concentration.

Determination of Hexose-6-phosphates. Glc6P, Fru6P and Man6P were measured sequentially by the addition of G6PDH, PGI and PMI to the assay mixture. Some batches of PMI contain PGM activity, therefore PGM was added before PMI to remove any Glc1P present which might otherwise give an overestimate of Man6P. In this case EDTA, an activator of PGM, was

added at a final concentration of 0.1 mM.

Determination of Free Hexoses. Glucose was measured by the addition of HK after the measurement of Glc6P. The addition of PGI gave fructose and the addition of PMI, mannose and Man6P. The free fructose and mannose were then obtained by subtracting the Fru6P and Man6P readings in the absence of HK from those in its presence, respectively.

ATP Determination. ATP was measured, after Glc6P was assayed, by the addition of HK and glucose. The use of yeast HK makes the method specific for ATP compared to other nucleoside triphosphates; the specificity was poorer with hexokinase from animal sources (92).

Determination of Man1P. Advantage was taken of the differences in stability towards acid and alkali of hexose-1-phosphates and the reducing sugars (free hexoses and their 6-phosphates) (93). Hexose-1-phosphates are stable in alkali while acid hydrolysis releases an equivalent amount of phosphate and the respective free sugar. Reducing sugars on the other hand are relatively stable in dilute acid whereas treatment with alkali results in a complex reaction with the formation of various degradation products, among others, phosphate, saccharinic acids and lactate.

In the determination of Man1P, the reducing sugars were first destroyed by boiling in a water bath for 30 min in 0.05 N KOH. Acid was then added, HCl or H_2SO_4, to a concentration of 0.1 N, the pH checked with pH paper, and hydrolysis was conducted for 20 min in a boiling water bath. A large amount of glucose was found after the acid hydrolysis (none was detected after the treatment with alkali), originating from Glc1P and UDP-Glc.

Neither free hexoses nor hexose-6-phosphates were detected in the sample after the treatment with alkali. After the subsequent acid hydrolysis, Glc6P and Man6P were detected. Presumably they represent the respective 1,6-bisphosphates that were originally present in the sample. Free glucose and mannose that were detected after the subsequent acid hydrolysis originated from the respective 1-phosphates.

The sequence of events is shown below:

$$\left\{\begin{array}{l}\text{Hexose}\\ \text{Hexose-1-P}\\ \text{Hexose-6-P}\\ \text{Hexose-(1,6)}P_2\\ \text{UDP-Glc}\end{array}\right\} \xrightarrow{OH^-} \begin{array}{l}\text{Hexose-1-P}\\ \text{Hexose-(1,6)}P_2\end{array} \xrightarrow{H^+} \begin{array}{l}\text{Hexose}\\ \text{Hexose-6-P}\end{array}$$

After acid hydrolysis, assays were carried out with and without hexokinase. Man1P was determined from the difference of the total (mannose + Man6P) found in the presence of HK and of Man6P alone when HK as omitted.

Assay of GDP-mannose. Two methods were tried in an attempt to measure GDP-Man in rat tissues. The experiments were done on extracts of rat liver which, because of its size, might contain a readily detectable level of the nucleotide.

In the first method the following strategy utilizing a combination of chromatographic techniques was tried. Nucleoside monophosphates and the sugar nucleotides were separated from the nucleoside di- and triphosphates by chromatography on hydroxyapatite (HA), the polyphosphates were retained on the HA column whereas the monophosphates and sugar nucleotides were eluted with 1 mM potassium phosphate buffer pH 6.8 (94). The sugar nucleotides were then separated from the monophosphates on an

anion exchange column, AG1-X4 (Cl^-), with a $CaCl_2$ gradient (95). The nucleotides were further characterized by ion exchange TLC on PEI-cellulose (96).

In a typical experiment a neutralized PCA extract from 6 g liver, diluted to about 60 ml, was applied onto an 8 ml column of HA in a 10 ml disposable syringe. After the sample passed through, the column was washed with the phosphate buffer at a rate of about 1 ml/min. About 25 ml buffer was used; however, the amount and flow rate were not critical as the purpose was to collect the whole eluate and buffer wash until the absorbance became negligible. The combined wash and eluate was applied directly onto a 10 x 0.8 cm column of AG1-X4 (Cl^-) and allowed to pass through at a rate of 0.5 ml/min. The column was then washed with 5 mM HCl until the absorbance became negligible (< 0.02), when a $CaCl_2$ gradient was started. The mixing chamber contained 200 ml of 5 mM HCl and the reservoir 200 ml of 0.2 N $CaCl_2$ in 10 mM HCl; fractions of about 4 ml were collected. GDP-Man was expected to elute towards the end of the gradient. The whole operation was carried out in a cold room.

The fractions that were thought to contain GDP-Man were combined and neutralized with a suspension of $Ca(OH)_2$, concentrated by vacuum on a rotary evaporator (below 40^0C) and lyophilized. To the dried material was added cold ethanol/ether (1:1, v/v), and the sample was kept in a freezer overnight. $CaCl_2$ remained in solution whereas the calcium salt of the nucleotide was precipitated and centrifuged out. This calcium salt was dissociated by the addition of ammonium oxalate and the calcium oxalate was centrifuged out.

The nucleotide was applied to a PEI-cellulose layer, along with nucleotide standards, and developed (ascending) with either 1 M $LiCl_2$

or 2 M Na-formate pH 3.4 (96). The nucleotide spots were detected by examining under short-wave UV light.

In the second method it was hoped to measure GDP-Man directly by following its oxidation to GDP-mannuronic acid; the reaction, catalyzed by GDP-Man dehydrogenase, consumes 2 mol NAD per mol GDP-Man oxidized.

An attempt was made to isolate the enzyme from A. viscosus according to Preiss (69,97). The bacteria (kindly supplied by Dr. L. K. Nakamura of the U.S.D.A., Northern Regional Research Center) was grown in several 1 l batches in a medium of 3% glucose, 0.3% casein hydrolysate, 0.4% K_2HPO_4, 0.08% $MgSO_4.7H_2O$, and 0.005% $MnSO_4$ (adjusted to pH 7.0 with H_2SO_4). After 16 to 24 h incubation at 28^0C, the cells were harvested by centrifuging the culture at 3^0C for 1 h at 10 000 x g. The bacterial precipitate was washed with cold 0.9% NaCl until free of polysaccharide. The cells were then stored in the freezer (-12^0C) until used.

The frozen cells were suspended in 3 volumes of 50 mM Tris buffer pH 7.5, containing 5 mM glutathione (GSH) and 10 mM $MgCl_2$. The cells were disintegrated by sonication for 30 sec using a Raytheon sonicator; the treatment being repeated 10 times.

All operations were carried out in the cold. The cell debris was centrifuged off at 30 000 x g for 10 min and the supernatant recentrifuged for 1 h at 105 000 x g. To the supernatant (105 000 x g) from 15 g cells was added 34 ml of 1% protamine sulfate, with continuous stirring, and after 10 min the suspension was centrifuged for 10 min at 26 000 x g. The supernatant was discarded and the precipitate extracted twice with 43 ml 0.3 M K-phosphate buffer (pH 7.0, 10 mM GSH). To the combined extracts (80 ml) was added 34 g solid ammonium sulfate; the dehydrogenase precipitates. The suspension was centrifuged for 10 min

at 26 000 x g and the precipitate dissolved in 20 mM K-phosphate buffer (pH 7.0, 10 mM GSH). The solution was then dialyzed overnight against 500 ml of phosphate buffer/GSH.

Calculations

The concentrations of hexose-6-phosphates and mannose-1-phosphate were corrected for the amount of a known addition of the respective metabolites being recovered from each tissue sample; the calculations were as shown in the Appendix. Recovery determinations were not attempted on ATP and the free hexoses. No attempts were made to correct for the contamination of the tissue sample by blood.

CHAPTER III

RESULTS

Fluorometer Performance

The fluorometer is a sensitive instrument; a great deal of effort was taken to eliminate or minimize noise. The most troublesome causes are specks of dust or paper fibers; filtration of all solutions, including enzymes, through a 0.45 micron Millipore filter solves the problem. To avoid introduction of dust into filtered solutions, a Cornwall syringe and Eppendorf pipets were used for transfer; ordinary pipets were never used. Kimwipes should never be used; lens paper was used instead.

The earlier part of this study was frustrating because of slow and erratic response of the fluorometer; the data obtained had to be discarded. Only much later was it suggested[1] that the problem might be due to magnetic influence on the photomultiplier tube; the problem was then solved by demagnetization of the tube using an AC magnetizer/demagnetizer (Brookstone Co, Worthington, MA)[2].

Preparation of Tissue for Analyses

Originally tissues were obtained from live animals frozen in situ

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[1] Joanna Ledford, personal communication.

[2] Supplied by Mr. Troy Barnes.

in liquid nitrogen. However, the results obtained were variable, with evidence of anoxic changes, namely, increase of Glc6P in the liver and a decrease in brain. The procedures mentioned under "Materials and Methods" (methods II and III of Table I and method III of Table II) were therefore adopted which, with consistency in their execution, probably gives a valid measure of the concentration of metabolites in vivo.

The results obtained for liver, kidney and testes are shown in Table I. There is essentially no difference between the values obtained after immersion into liquid nitrogen of the individual tissue (liver) or of the whole animal with the abdominal cavity exposed. Anoxic changes are obvious in the tissues obtained by immersion of the live animals.

Freeze-clamping of liver and kidney was tried on several occasions; the concentrations, in the liver of a 100 g rat, of Glc6P, Fru6P and Man6P were 463, 141 and 122 nmol/g, respectively. The tool used was a vise-grip sheet metal clamp obtained from a local hardware store and modified in the University's physical shop to increase its cooling capacity by attachment of aluminum blocks.

The values above, indicating anoxic changes, were from liver clamped after securing blood from the tail vein. Better results might be obtained with practice and without such manipulation. However, the method was abandoned because of the tedious procedure for obtaining the tissues, especially the kidney, from which the adhered perirenal fat has to be removed from the frozen tissue.

Brain, which presents a special problem because of its anatomy, could not be frozen within a reasonable time by immersion, except with smaller animals. The best approach to date is freeze-blowing (98) whereby air under pressure enters the cranial cavity through a steel probe and

the supratentorial portion of the brain is forced out through another probe into a chamber precooled in liquid nitrogen. Siesjö et al. (99) described the freezing of brain in situ after exposing the skull by surgery under anesthesia; the animal was kept ventilated during the freezing process which takes several minutes to complete.

The concentration of Glc6P, Fru6P and Man6P in brains from 75 to 150 g rats are shown in Table II. The concentrations of these metabolites are lowest in brains from animals frozen by immersion in liquid nitrogen or decapitated and the severed head dropped directly into the refrigerant. The level of Glc6P obtained by the procedure adopted in this study (method III of Table II) is comparable to that obtained by freezing of the brain in situ after surgical exposure (99). The highest values are from brains obtained by freeze-blowing[3].

The possibility of storing tissues in the deep-freeze ($-70^{0}C$) with later processing at a convenient time was investigated. Contrary to the statement that tissues could be thus stored safely for several weeks (100), it was found that liver Glc6P and Fru6P decreased, after one week, by about 20%, while Man6P was essentially unaltered.

Hexose-6-phosphates

The concentration of hexose-phosphates was calculated without correction for contamination by blood; recoveries (for calculation see Appendix) varied between 73 and 97%. The results for the hexose-phosphates as obtained by the procedures mentioned above are within the normal values obtained by freeze-clamping or by immersion as compiled

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[3]Thanks to Drs. R. L. Veech and T. King for the brain sample.

TABLE I

CONCENTRATIONS OF HEXOSE-6-PHOSPHATES
IN LIVER, KIDNEY AND TESTES

Tissue	Method*	Glc6P**	Fru6P	Man6P
Liver	I (3)	490 ± 68	81 ± 9	130 ± 13
	II (3)	278 ± 27	70 ± 20	97 ± 22
	III (9)	294 ± 10	72 ± 5	99 ± 6
Kidney	I (2)	25	10	13
	III (6)	42 ± 4	12 ± 2	29 ± 4
Testes†	III (3)	32 ± 1	8 ± 0.6	3 ± 1

*Number of observations in brackets.
Method I : Immersion of live animal into liquid nitrogen.
Method II : Rats anesthetized with ether, the abdominal cavity exposed and the whole animal immmersed in refrigerant.
Method III: As in method II, the tissue excised and plunged in the refrigerant.

**Results are in nmol/g (± S.E.), corrected for recoveries, from 200 - 250 g female rats.

†From 350 g males.

TABLE II

CONCENTRATION OF HEXOSE-6-PHOSPHATES
IN RAT BRAIN

Method*	Glc6P†	Fru6P	Man6P
I (3)	48 ± 3.5	10 ± 1.2	23 ± 3
II (3)	39 ± 0.3	12 ± 0.6	21 ± 2
III (2)	92 ± 1	19 ± 1	21 ± 4
IV (3)	134 ± 12	31 ± 5	51 ± 3

*Number of observations in brackets.
Method I : Immersion of live animal into liquid nitrogen.
Method II : Rats anesthetized with ether and the head decapitated into liquid nitrogen.
Method III : Same as I, except the skin of the forehead was removed under ether anesthesia.
Method IV : Freeze-blown samples obtained from Drs. Veech and King, kept in deep-freeze (-70^0C) several weeks.

†Results from 75 to 150 g rats. Values are in nmol/g (± S.E.), corrected for recoveries.

by Williamson and Brosnan (101)

Suckling rats were used to provide tissue samples that could be quickly frozen without undue manipulation or stress; the results are shown in Table III. A comparison of these levels of metabolites with those obtained from older rats reveals some significant differences. The concentration of Glc6P in suckling rats is higher in the kidney and brain and lower in liver. The values obtained from the suckling rats are comparable to those obtained from freeze-blown brains of older animals. The young and older rats have similar Fru6P levels in brain and kidney. However, liver Fru6P is higher in the older age group.

As far as we are aware this is the first study of Man6P and Man1P in animal tissues other than the brain. The concentrations of Man6P in brain obtained by freeze-blowing and that of sucklings are similar; these values are higher than those obtained by other methods but significantly lower than that reported by Sloviter et al. (1). Older rats have higher concentrations of Man6P in liver. The two age groups have similar values for kidney.

The report that testes contains neo-inositol, which is derived from Man6P (81), prompted a study of that organ. The results, obtained from 350 g rats, are presented in Table I.

Mannose-1-phosphate

There has been no report on the determination of Man1P in animal tissues. Since the concentration is expected to be low, the lack of a specific enzymatic assay system presents a technical problem. Free and other phosphorylated sugars which are present in much higher concentrations must first be removed before Man1P is determined, after hydrolysis,

TABLE III

CONCENTRATION OF HEXOSE-6-PHOSPHATES
IN TISSUES OF SUCKLING RATS

Tissues	Glc6P	Fru6P	Man6P
Brain	131	17	52
	135	20	41
Liver	136	24	54
	175	32	48
Kidney	83	13	32

Rats 2 weeks old (20 g) were used, frozen by immersion. The values are from individual experiments, in nmol/g; those for brain and kidney are from 2 or 3 rats combined.

as mannose or phosphate.

Several methods have been tried, giving similar results (see Table IV) with recoveries varying between 60 and 110%. The first method removes mannose, fructose, glucose and their 6-phosphates by enzymatic conversion to 6-phosphogluconate (see diagram under "Methods"). Incubations were carried out at 30^0C and aliquots were tested at intervals for the completeness of the reaction. The reaction was quenched with either PCA or H_2SO_4. ManlP was then determined after hydrolysis with alkaline-phosphatase according to Grassl (102) and the liberated mannose assayed as mentioned previously. Hydrolysis can be done chemically using 0.1 N H_2SO_4 and boiling in a water bath for 20 min. Free mannose is assayed enzymically after neutralization and precipitation of sulfate as $BaSO_4$.

The second method is based on the principle that reducing sugars, namely hexoses and their 6-phosphates, can be converted to the corresponding polyols by reduction with $NaBH_4$; hexose-1-phosphates are not affected and may be determined as above. Reduction was carried out with $NaBH_4$, twice the weight of the reducing sugars present, at room temperature and pH 10 for 2 hours (103). The solution was neutralized with HCl, thus destroying excess borohydride, cations were removed with AG50-X4 (column or batchwise) and borate removed by repeated evaporation with methanol (water pump).

The two methods described above are involved and not practical for analysis of multiple samples. It was found much simpler and more convenient to destroy reducing sugars by alkaline hydrolysis followed by acid hydrolysis of the hexose-1-phosphates (see "Methods"). The solution contains alcohol and tends to bump during alkaline hydrolysis. Loss of sample is avoided by carrying out the hydrolysis in an Erlenmeyer flask

TABLE IV

MANNOSE-1-PHOSPHATE CONTENT OF RAT TISSUES

Experiment #	Tissue	Treatment	Concentration	Recovery %
1.*	Liver	none	18	72
2.*	Liver	none	16	77
3.†	Liver	none	15	nd
4.	Liver	none	13	110
	Kidney	none	12	110
5.	Brain	none	11	60
	Liver	M-15¶	100	60
	Kidney	M-15	79	60
6.	Brain	M-15	15	92
	Brain	M-60	14	92
7.	Liver	G-15	14	83
	Liver	G-60	16	83
	Testes	none	3	83

Results are in nmol/g of tissue, corrected for recoveries, representing individual values from 110 to 300 g female rats, except testes as the average from 3 males weighing 350 g.

ManlP was determined after alkaline destruction of reducing sugars, except:

*removal of reducing sugars by borohydride reduction.

†removal of reducing sugars by enzymic conversion to 6-phosphogluconate.

¶M-15, G-15, etc. indicate time in minutes after mannose or glucose injection.

or a large test tube. A graduated centrifuge tube is used for hydrolysis with either H_2SO_4 or HCl. H_2SO_4 is neutralized with $Ba(OH)_2$ and $BaSO_4$ is removed from the sample by centrifugation. Excess $Ba(OH)_2$ must be avoided because the subsequent assay uses enzyme solutions containing ammonium sulfate and the $BaSO_4$ so formed results in turbidity of the assay solution. HCl may be used for the hydrolysis and neutralized with KOH; the KCl formed has no adverse effect on the enzymatic reactions needed for mannose determination.

The acid hydrolysate is decolorized with charcoal before neutralization; decolorization is not as effective if carried out after neutralization. The fact that the hydrolysate became colored, presumably because of the presence of amino acids in the tissue extract, raised the question as to whether or not this would have any untowards effect on the assay. Standard solutions containing the expected level of free hexoses and their 6-phosphates along with a mixture of amino acids were hydrolyzed according to the above procedure; no free sugars were detected in the alkaline or acid hydrolysate. Mannose-1-phosphate similarly treated was quantitatively recovered. Removal of amino acids with cation exchanger, AG50-X4 (H^+), prior to hydrolysis resulted in lower recoveries of Man1P.

Of concern was whether GDP-Man, if present, would contribute to the mannose measured after acid hydrolysis. However, no mannose was detected when a known solution of GDP-Man was put through the above procedure. Apparently mannose is released on treatment of GDP-Man with alkali and thus degraded. Similar treatment of UDP-Glc, which differs from GDP-Man only at the hydroxyl group on C_2 of the hexose, resulted in the detection of glucose in the final acid hydrolysate.

The identity of hexose-1-phosphate present in the alkaline hydrolysate was verified by passing the solution through a 4 x 1 cm column of the anion exchanger AG1-X4 (Ac^-). After the hydrolysate (equivalent to 5 g liver) was passed through, the column was washed with 10 ml of water followed by 30 ml of 0.01 N HCl and the sugar-phosphates eluted with 0.03 N HCl. 3 ml fractions were collected; the sugar-phosphates came off in tubes 4, 5 and 6. Hexose-1-phosphates, and any 6-phosphates present, would come off together; the fractions were conveniently assayed for the presence of Glc1P with PGM and G6PDH. Hexose-6-phosphates were not detected in any of the fractions. A sample of the hexose-phosphate peak was acid hydrolysed and assayed for glucose, mannose and inorganic phosphate (104); the ratio of (glucose + mannose) : P_i was 0.8 : 1. This lack of 1 : 1 stoichiometry could be due to the presence of other phosphorylated compounds that were not separated by the ion-exchange procedure.

Response to Sugar Loading

Sugar loading, by intraperitoneal injection at a dose of 20 mmol per kg of body weight of either glucose, fructose or mannose, was carried out to see what changes it would cause in the concentrations of Man6P and Man1P. The method for Man1P determination was developed later in the course of the study, thus fewer results are presented for Man1P. The levels of hexose-phosphates were determined 15,30 and 60 min after the injection of the respective sugars; the results are shown in Tables V, VI and VII. The available data indicate a similar pattern of response between the younger (60 to 100 g) and older animals (150 to 300 g).

Glucose loading. Liver Glc6P was decreased in agreement with the

observations of Hers (105); the effect occurred at 30 min after the injection. After 60 min the Glc6P level appeared to rebound to a higher than normal value. The same pattern was observed with liver Fru6P; the levels of Glc6P and Fru6P, 60 min after the injection, appeared to be significantly higher than control values (Table V). With these exceptions, glucose loading had little effect on hexose-phosphate concentrations.

Fructose loading. An increased level of liver hexose-6-phosphates was observed, with the concentrations at 60 min being definitely higher than controls (Table VI). In the kidney, peak values (above controls) of the 6-phosphates were observed at 30 min. In brain, the levels of Glc6P and Man6P, 60 min after the injection, appear to be higher than those of controls while Fru6P remains constant throughout the study.

Mannose loading. Liver Man6P was increased by 4 to 40 times that of control (Table VII) and a definite increase of Glc6P and Fru6P was also observed 60 min after the injection. Man6P in brain had increased 15 min after the injection and remained elevated during the 60-minute period. In kidney, the level of Man6P appeared elevated at 30 min, but normal at 15 and 60 min. Fru6P remained within the normal range in brain and kidney. In all tissues elevated values of Man1P were obtained; liver and kidney Man1P rose to 7 to 10 times the control levels. Liver Man1P also increased after fructose injection (Table VI) but not after glucose loading (Table V).

ATP and Free Hexoses

Initially we had no interest in determining these metabolites. However, a report concerning the toxicity of mannose to honeybees (106)

TABLE V

EFFECT OF GLUCOSE LOADING ON HEXOSE-PHOSPHATES IN RAT TISSUES

Tissue	Glc6P*		Fru6P		Man6P		Man1P	
Liver								
Control**	294		72		99		15	
G-15§	233	-	50	-	84	-	-	-
	360	-	80	-	88	-	14	-
G-30	149	332	47	62	100	80		
	92	-	39	-	107	-		
	77	-	37	-	93	-		
G-60	575	-	126	-	126	-	16	-
Kidney								
Control	42		12		29		12	
G-15	74	-	18	-	43	-		
G-30	39	43	14	10	48	36		
	-	69	-	14	-	26		
Brain								
Control	92		19		21		11	
G-15	-	75	-	15	-	36		

*Each value in nmol/g represents individual determinations. Those at the left of each column are from 150 - 300 g rats, those at the right are from 60 - 100 g rats.

**Control values are from Tables I, II and IV.

§G-15, etc indicates time, in minutes, after glucose injection.

TABLE VI

EFFECT OF FRUCTOSE LOADING ON HEXOSE-PHOSPHATES IN RAT TISSUES

Tissue	Glc6P		Fru6P		Man6P		Man1P	
Liver								
Control	294		72		99		15	
F-15*	272	-	71	-	106	-	75	-
F-30	327	271	101	73	110	73		
	305	230	93	61	129	107		
	470	363	110	89	117	78		
F-60	842	-	143	-	154	-	42	-
Kidney								
Control	42		12		29		12	
F-15	77	-	14	-	28	-	12	-
F-30	176	159	53	35	115	74		
	225	-	52	-	106	-		
F-60	65	-	12	-	24	-	32	-
Brain								
Control	92		19		21		11	
F-15	60	71	16	15	24	30	15	-
F-30	-	80	-	16	-	32		
	-	87	-	19	-	24		
F-60	116	-	20	-	41	-	14	

*F-15, etc. indicates time, in minutes, after fructose injection.

For other explanations, see footnotes to Table V.

TABLE VII

EFFECT OF MANNOSE LOADING ON HEXOSE-PHOSPHATES IN RAT TISSUES

Tissue	Glc6P		Fru6P		Man6P		Man1P	
Liver								
M-15*	305	202	73	57	410	2620	-	-
	247	-	56	-	930	-		
	398	-	96	-	1910	-		
	297	-	58	-	440	-	100	-
	348	-	78	-	1220	-	134	-
M-30	354	262	89	52	4310	793		
	311	274	57	64	1273	942		
M-60	540	-	120	-	1560	-	186	-
	506	-	110	-	1010	-		
Kidney								
M-15	58	-	10	-	44	-		
	18	-	3	-	24	-	79	-
	21	-	9	-	23	-		
M-30	38	72	13	13	107	82		
	57	-	12	-	53	-		
M-60	54	-	11	-	32	-		
Brain								
M-15	130	-	20	-	130	-		
	56	76	17	16	85	162	28	-
	38	-	19	-	128	-		
M-30	-	95	-	21	-	112		
	-	45	-	14	-	195		
M-60	95	-	22	-	58	-		

For control values and other explanations, see Table V.

*M-15, etc. indicates time in minutes after mannose injection.

in which the depletion of ATP was suggested as the underlying cause (107) prompted us to determine the ATP levels in rat tissues. The determination of free hexoses was carried out to see the changes introduced by hexose loading and to correlate them with any changes in the phosphorylated intermediates.

The results are presented in Tables VIII and IX. Individual values are presented together with the corresponding levels of Glc6P and Man6P for the purpose of making correlation with the changes noted in the preceding tables. The levels of ATP in the tissues of control animals were lower, by about 25 to 30%, than those of reported values (101). Control glucose levels are within the reported normal range in both liver and brain.

The concentration of ATP in the blood of the control animal is within the reported normal range and remains essentially unaltered by the injection of either of the hexoses, glucose, fructose or mannose. An increase in blood glucose is observed not only after the injection of glucose, but also after mannose and fructose loading.

The tissue content of ATP decreases after the injection of fructose or mannose with a trend of returning to normal after 60 minutes; brain ATP is not significantly affected by mannose loading. Liver ATP also does not appear to differ from that of control following the injection of glucose. In the determination of ATP using hexokinase and G6PDH caution is necessary since mannose would compete in the hexokinase reaction. Therefore, PGI and PMI have to be added to the ATP assay and the proper corrections applied.

The sugars injected into the animals are rapidly excreted by the kidney, as evidenced by the very high levels of fructose or mannose

TABLE VIII

ATP AND FREE HEXOSES IN RAT TISSUES AFTER GLUCOSE AND FRUCTOSE LOADING

Tissue	Rat #	Treatment	ATP	Glc	Fru	Man	Glc6P
Blood	1	control	0.40	4.77	0	0	nd
	2	G-15	0.47	19.9	0	0	nd
	3	G-60	0.46	12.4	0	0	nd
	4	F-15	0.44	7.70	7.15	0	nd
	5	F-60	0.55	4.58	1.89	0	nd
Liver	6	control	1.56	6.74	0	0	225
	7	control	nd	7.0	0	0	301
	8	G-15	1.91	11.3	0	0	360
	9	G-60	1.58	22.6	0	0	575
	3	G-60	1.82	9.5	0	0	nd
	10	F-15	0.68	7.8	8.8	0	272
	11	F-60	1.18	9.03	1.06	0	842
Brain	6	control	1.58	1.12	0	0	93
	10	F-15	1.05	2.81	1.17	0	60
	11	F-60	1.75	2.22	0.13	0	116
Kidney	10	F-15	0.84	5.4	29.2	0	77
	11	F-60	0.88	5.09	1.13	0	65

Results are from rats weighing 200-300 g; all values are in µmol/g (or µmol/ml in blood), except Glc6P in nmol/g.

nd : not determined

TABLE IX

ATP AND FREE HEXOSES IN RAT TISSUES
AFTER MANNOSE LOADING

Tissue	Rat #	Treatment	ATP	Glc	Fru	Man	Man6P
Blood	12*	M-15	nd	3.0	0	5.6	nd
	13	M-15	0.48	5.79	0	12.0	nd
	14	M-60	0.44	4.2	0	7.35	nd
Liver	15	M-15	0.97	8.83	0	15.37	1910
	16	M-15	1.33	9.3	0	14.1	440
	17	M-15	nd	8.6	0	27.5	1220
	18*	M-15	nd	6.2	0	11.0	930
	19*	M-30	nd	6.7	0	10.6	942
	20	M-60	nd	15.2	0	24.7	1560
	21	M-60	1.70	4.7	0	6.9	1010
Brain	15	M-15	2.13	2.46	0	3.94	128
	16	M-15	nd	1.33	0	2.29	85
	18*	M-15	nd	2.2	0	3.8	130
	19*	M-30	nd	2.1	0	4.5	195
	22	M-60	1.96	1.94	0	1.8	58
Kidney	15	M-15	0.78	3.97	0	35.12	23
	16	M-15	0.71	4.1	0	35.0	24
	22	M-60	1.16	4.23	0	15.1	32

See Table VIII for explanations.

*Rats weighing 75 to 170 g.

in this organ. The tissue concentration of glucose appeared to increase slightly after the injection of either fructose or mannose. The increase of fructose in the tissues after fructose loading does not reach as high a level as that of mannose after mannose loading. Fructose levels in the tissues decrease 60 min after fructose loading while tissue glucose remains normal or slightly higher (Table VIII), resulting in an increase of the glucose : fructose ratio. On the other hand, the concentration of mannose in the tissues after mannose injection remains higher than that of glucose, even 60 min after injection; the liver mannose : glucose ratio is generally 1.5 to 1.8. Brain mannose appears to be higher at 30 min after injection and the mannose : glucose ratio changes from about 1.7 at 15 min to 2.4 (30 min) and 0.93 (60 min).

GDP-Mannose

Attempts to detect and determine GDP-Man in rat liver were unsuccessful. The chromatographic method works well with standard solutions: GDP-Man was well separated from ATP and ADP on a hydroxyapatite column, and from AMP on subsequent elution from a column of AG1-X4. The identity of GDP-Man was further verified by TLC on PEI-cellulose and from the characteristic absorption spectrum of the guanosine moiety (108). GDP-Man was also hydrolyzed by boiling (water bath) for 10 min in 0.01 N HCl, resulting in the release of mannose. An eluate of liver extract (HA eluate with 0.01 N K-phosphate buffer pH 6.8) which was subsequently developed on an AG1-X4 column showed several ultraviolet absorbing peaks, but GDP-Man was not detected in any of the fractions.

The isolation of GDP-Man dehydrogenase from A. viscosus was also unsuccessful. It was learned later that the bacteria had undergone

mutation (Dr. Jack Preiss, communication with Dr. Koeppe).

Recently, Sudarat Manochiopinig in our laboratory has found this enzyme in cultures of A. viscosus obtained from Dr. Preiss.

CHAPTER IV

DISCUSSION

Sampling Procedure

The preparation of tissues for the study of metabolite levels requires rapid "freezing" of metabolic processes, either by the use of refrigerants such as liquid nitrogen or Freon cooled with liquid nitrogen or by microwave heating. Several variants of this basic principle, directed towards the arrest of anoxic/ischemic changes, have been reported in the literature. The animals used may be anesthetized or not; however, no single procedure applicable for obtaining different tissues from an individual animal has been described. The simplest procedure, of freezing the whole animal in a refrigerant, is not satisfactory except for very small, young animals.

The best method of obtaining brain samples is by freeze-blowing (98), although this method does not allow regional studies to be done. Microwave irradiation has been shown to give results different from those obtained by freeze-blowing. The differences were not attributable simply to anoxia. Rather, the changes seem to reflect the different thermal characteristics of the various enzymes which must be denatured to stop metabolism of the substrate to be measured (109). A study of the changes in phosphocreatine, ATP and lactate showed that fixation of brain by immersion in liquid nitrogen approximates that by freeze-blow-

ing more closely than does microwave irradiation (110).

Swaab (111) demonstrated that the skin of the skull was the most important insulator; cortical temperature would drop to 0^0C in 3.8 sec if the skin was removed compared to 30 sec if the skin was left intact. The initial cooling with liquid nitrogen was as fast as with Freon at its melting point, but eventually cooling with liquid nitrogen was faster. Mouse brain 6 mm below the skull reached -40^0C within 20 sec (intact head, 112).

Other organs can be quickly frozen by freeze-clamping between two metal plates chilled to liquid nitrogen temperature. Since this method is not always practical or convenient for all organs, satisfactory results may be obtained by quickly removing a small piece of tissue from an anesthetized animal and immersing it in liquid nitrogen. Alternatively the tissue can be exposed, under ether anesthesia, and flooded with the refrigerant (100).

The methods mentioned necessitate the use of anesthetics, thus introducing another variable while solving the problem of easy access to the desired tissues. Anesthetics in general cause a depression of metabolic rates. There was no significant alteration in the level of adenine nucleotides of brain under anesthesia with phenobarbital while glucose and Glc6P were higher than the control values (99). Dale (113) cosidered nembutal unsatisfactory because of the long induction period and ether anesthesia preferable to represent the concentration of metabolites in vivo. However, ether will elicit a sympathetic response, causing the release of catecholamines, with the concomitant increase in glycogenolysis and lipolysis (114).

With so many variables involved it is not surprising to see a wide

range of control values reported in the literature (100); representative values are presented in Table X. Faupel et al. (115) introduced the double-hatchet method for sampling rat liver whereby the animal was cut in one stroke at the neck and just below the chest. The method obviates the use of anesthetics; liver samples could be obtained and frozen between cooled aluminum blocks within 3.5 sec.

In the present study rats anesthetized with ether were used so that different tissue samples could be obtained from one animal. The levels of Glc6P and Fru6P in liver and kidney are within the reported range of normal values, while those of brain are lower than those obtained by freeze-blowing but in agreement with the values obtained by freezing in-situ with the skull exposed. The lower concentration of ATP obtained in the limited number of control samples suggests that anoxic changes may have occurred, at least in some samples.

There is room for improvement in the sampling procedure and a modification of the double-hatchet method seems most promising for obtaining multiple samples from a single animal. This would be preferable to the usual procedure of obtaining different tissues from a number of animals because a better appraisal of the effect of a stimulus (e.g. mannose loading) on the whole animal could be obtained. A multiple hatchet that would cover the whole length of the animal, with the blades separated by about 5 mm or less, would serve the purpose. The animal is placed in a chamber (disposable, if desired) in which it has been acclimatized to minimize or avoid any stressful effect and the hatchet is struck down on the unanesthetized animal which is then immediately covered with liquid nitrogen. The freezing process would be faster using a precooled hatchet, in which case the blades should be made of metal which remains

TABLE X

CONCENTRATION OF METABOLITES REPORTED IN THE LITERATURE

Tissue	Glc6P	Fru6P	ATP	Ref.
Brain	95	nd	3.0	99*
	116	nd	2.45	109**
	159	51	2.36	116¶
Liver	153	33	nd	117†
	160	50	2.74	118§
	278	76	3.4	115
	229-253	33-40	1.89-2.01	119§§
	195-370	nd	nd	101
Kidney	39	13	1.43	118§

*Freezing in-situ, halothane anesthesia for 60 min.

**Whole body immersion, unanesthetized.

¶Freeze-blown, pentobarbital anesthesia for 20 min.

†Freeze-clamp in-situ, pentobarbital anesthesia.

§Cervical dislocation, open abdomen, tissue excised and freeze-clamped.

§§Freeze-clamp in-situ, ether anesthesia

sharp under the freezing and warming stress.

It is desirable for statistical purposes to obtain and prepare the extract of the different tissues from the control and the variously treated animals on the same day. Because it is not possible to assay these extracts immediately, it is necessary to determine the stability of the metabolites in stored extracts.

Mannose-phosphates and Related Metabolites

This study describes the presence of Man6P and ManlP in rat tissues: brain, liver, kidney and testes. The concentration of Man6P in brain of about 20 to 50 nmol/g is much lower than that reported earlier (about 320 nmol/g) by Sloviter et al. (1). Except for testes, the levels of Man6P are higher than those of Fru6P with the ratio of Man6P : Fru6P in the range of 1.4 to 3, which is not far removed from the equilibrium value of about 1.0 reported for the reaction catalyzed by yeast PMI (76). The concentration of Man6P in liver appears to be slightly increased after glucose injection. The rise in liver Man6P is definite 60 minutes after fructose loading, with the ratio Man6P : Fru6P remaining essentially constant (Tables I - VI).

The concentration of Man6P, following mannose loading, is increased in the liver, brain and kidney. Liver Man6P varies between 4 and 40 times that found in controls and is accompanied by a similar increase of ManlP. Brain Man6P is about 3 to 10 times control values during the 60-minute period after mannose loading, while kidney Man6P appears to reach a peak of 2 to 3 times that of control 30 min after the injection. Although the data are limited, an increase of ManlP is also apparent in kidney and brain. These observations suggest the interconversion of Man6P and

Man1P in rat tissues. It is still unknown what enzyme, if any, is involved in the interconversion because PMM has so far not been detected in rat tissues[1].

The phosphorylation of mannose to Man6P could be carried out by the enzymes hexokinase or glucokinase. Glucokinase activity is known to be decreased by diabetes or fasting (120) and it would be of interest to follow the effect of mannose loading in these conditions.

A fall in ATP is observed after fructose or mannose loading; the decrease of liver ATP correlates quite well with the increase of Man6P. This fall of ATP after mannose injection is similar to that after fructose, sorbitol or glycerol loading (121), in which the formation of phosphorylated products is followed by a rate limiting step. Brain ATP appears to be within the normal level, presumably because of the relatively low increase of Man6P.

GDP-Mannose

A look at the literature (54 - 61) reveals that a sizable quantity of materials were used for the isolation of GDP-mannose. Anion exchange chromatography was invariably used, and the separation of nucleotides required a large volume of eluant, a formate gradient in most cases. The values reported did not mention the possibility of losses occurring, among others, because of overlapping peaks; GDP-Man follows ADP in the formate system and elutes after ATP when a calcium chloride gradient is used (95).

In this study we wanted to quantitate the levels of GDP-mannose in

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[1]J. Ledford and S. Manochiopinig, personal communication.

tissues, starting with a small amount of material. Clearly, the methods above are not directly applicable for this purpose. The fact that hydroxyapatite retains nucleotide di- and tri-phosphates and not sugar nucleotides (94) appears useful for the pretreatment of tissue extracts before fractionation on an ion exchange column. This was verified by using known solutions of nucleotide mixtures. However, we failed to detect any GDP-mannose in liver extracts. This failure might be attributable to a low level of GDP-Man in liver and possible degradation during the acid extraction process. It would be useful to try the above strategy on a convenient source such as egg-white, in which most of the nucleotide present is GDP-Man (56), or pig's milk (61)! Various procedures have been reported which might be useful as alternatives, or in addition to, the above strategy.

It is unfortunate that the enzymatic method, which seems to be more specific and convenient, could not be used for the time being, because of the mutation of the bacteria used as the enzyme source. Work is still being carried out in this lab to isolate the enzyme using a new batch of bacteria from Dr. Preiss (S. Manochiopinig).

CHAPTER V

SUMMARY

The determination of mannose-phosphates in rat tissues has been accomplished using an enzymatic assay coupled to the formation of NADPH, the fluorescence of which is measured. Mannose-6-phosphate is present in brain, liver, kidney and testes at concentrations of 21, 100, 29 and 12 nmol/g, respectively.

Mannose-1-phosphate is present in these tissues at concentrations ranging from 3 nmol/g in the testes to about 15 nmol/g in liver. The method for the determination of mannose-1-phosphate has been described in detail. Several procedures were tried for this determination, the most convenient being the enzymatic measurement of free mannose produced from mannose-1-phosphate by sequential alkaline and acid hydrolysis of tissue extracts.

Following intraperitoneal injection of mannose (20 mmol/kg body weight) the concentration of mannose-6-phosphate and mannose-1-phosphate is increased in liver and brain. Although phosphomannomutase activity has not been detected in mammalian tissue, these results suggest that mannose-6-phosphate may be the precursor of mannose-1-phosphate.

Attempts to measure GDP-mannose by chromatographic and enzymatic means failed; the rationale behind the approach and the probable causes of failure are discussed.

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APPENDIX

DETERMINATION OF RECOVERY AND CONCENTRATION OF METABOLITES

A tissue sample is divided into two portions, m gram is used for the determination of the metabolite X and n gram for the assay with a known amount, s, of a standard solution of X added. It is desired to determine the concentration of the metabolite per g of tissue, x, and the percentage recovery, p.

Let the amount of X found in m gram of tissue = M, and the amount in n gram (with s added) = N. The original concentration of X in the tissue, corrected for recovery is determined as follows:

$$M = xmp/100 \longrightarrow p = 100M/mx$$

$$N = p(xn + s)/100 \longrightarrow p = 100N/(xn + s)$$

Rearranging the equation would give:

$$(Nm - Mn)x = Ms$$

$$\text{and} \quad x = Ms/(Nm - Mn)$$

VITA 2

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Biographical:

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Education: Graduated from Methodist English School, Medan, Indonesia in May, 1961; received the Bachelor of Science degree (cum laude) in Medicine from the School of Medicine, University of Indonesia, Jakarta in 1964 and Doctor of Medicine from University of Indonesia in 1968; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in December, 1978.

Professional Experience: Employed by the Government of Indonesia as teaching staff and instructor at University of Indonesia, School of Medicine, Department of Biochemistry, Jakarta, Indonesia from 1967 to present; served as lecturer and instructor at JARSI Medical School, Jakarta from 1969 to 1973; served as lecturer and instructor at Trisakti University, School of Medicine, Jakarta from 1970 to 1971; served as lecturer at University of Indonesia, School of Public Health in 1972; served as research assistant at Oklahoma State University from 1975 to 1978; served as teaching assistant in 1974.

Professional Organization: Member of the Indonesian Biochemical Society.