STUDIES ON PHOSPHORYLATIVE PATHWAYS OF GALACTOSE METABOLISM IN RAT HEART AND BRAIN

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ABSTRACT

STUDIES ON PHOSPHORLATIVE PATHWAYS OF GALACTOSE METABOLISM IN RAT HEART AND BRAIN

By

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The ability of adult rat heart to metabolize D-galactose via the sugar-nucleotide and reductive pathways was studied. Rat myocardial slices oxidized D-[1-^{14}C] galactose to $^{14}CO_2$ at less than 5% the rate of D-[1-^{14}C] glucose oxidation. Furthermore, heart homogenates poorly converted D-[1-^{14}C] galactose into phosphorylated intermediates under conditions in which 25% of the labeled galactose was converted to metabolites by liver homogenates.

The Leloir pathway enzyme activities of rat heart were found to be present in amounts 8%, 38% and 23% that of rat liver for galactokinase, galactose 1-phosphate uridyl transferase and UDP-galactose 4'-epimerase, respectively. The corresponding values for the rat brain enzymes relative to liver were 27%, 11% and 120%, respectively. Michaelis constants for the three enzymes were comparable in heart, brain and liver.

The reduction of galactose to galactitol in perfused heart preparations was dependent on the perfusate galactose

concentration and appeared to be substrate saturable with a "Km" of 30 mM for galactose. The rate of galactitol production in perfused rat hearts was several times greater than the rate of galactose oxidation to carbon dioxide by heart slices at equivalent perfusion and incubation media galactose concentrations. Increasing the perfusate galactose concentration also had the effect of reducing myocardial ATP and creatine phosphate levels.

Rat heart and brain, but not liver, crude enzyme preparations produced what appeared to be galactose 6-phosphate in addition to galactose 1-phosphate when incubated with high concentrations of D-galactose [30 mM]. Tissue hexokinase activities were measured as a possible explanation for galactose 6-phosphate synthesis.

Galactose 6-phosphate was further identified in the brains of galactose-intoxicated chicks and rat hearts perfused with galactose by spectrophotometry and analysis by combined gas-liquid chromatography-mass spectrometry. Tissue concentrations of galactose 6-phosphate were compared to selected glycolytic and galactose metabolites. While galactose 6-phosphate levels were comparable to the levels of certain glycolytic metabolites, galactose 6-phosphate only comprised about 5% of the total galactose phosphate.

The subcellular distribution of $NADP^+$ and NAD^+-dependent glucose 6-phosphate and galactose 6-phosphate dehydrogenases were studied in rat liver, heart, brain, and chick brain. Only liver particulate fractions oxidized glucose 6-phosphate

and galactose 6-phosphate with $NADP^+$ or NAD^+ as cofactor. While all of the tissues examined had $NADP^+$-dependent glucose 6-phosphate dehydrogenase activity, only rat brain soluble fractions had $NADP^+$-dependent galactose 6-phosphate dehydrogenase activity. Rat liver microsomal and rat brain soluble galactose 6-phosphate dehydrogenase activities were kinetically different although their reaction products were both 6-phosphogalactonate. Rat brain subcellular fractions did not oxidize 6-phosphogalactonate with either $NADP^+$ or NAD^+ cofactors but phosphatase activities hydrolyzing 6-phosphogalactonate, galactose 6-phosphate and galactose 1-phosphate were found in crude brain homogenates.

The effects of galactose 6-phosphate and 6-phosphogalactonate on several glycolytic and hexose monophosphate shunt enzymes were investigated. 6-Phosphogalactonate was found to be a competitive inhibitor of rat brain 6-phosphogluconate dehydrogenase.

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By

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"Nature, my dear sir, is only a hypothesis."

--Raoul Dufy

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ABBREVIATIONS

ATP, ADP, AMP	Adenosine tri-, di-, or mono-phosphate
NAD^+, NADH	oxidized and reduced nicotinamide adenine dinucleotide
$NADP^+$, NADPH	oxidized and reduced nicotinamide adenine dinucleotide phosphate
UDP, UMP	uridine di- or mono-phosphate
UDPG	uridine diphosphate glucose
UDP-Gal	uridine diphosphate galactose
Gal 1-P	galactose 1-phosphate
Gal 6-P	galactose 6-phosphate
Tris	tris (hydroxymethyl) aminomethane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
6-P-Gala	6-phosphogalactonic acid
G6PD	glucose 6-phosphate dehydrogenase
H6PD	hexose 6-phosphate dehydrogenase
PPi	inorganic pyrophosphate
TCA	trichloroacetic acid
CPM	counts per minute

INTRODUCTION

Literature Survey

To supplement the discussion of the project's rationale and objectives, a background in mammalian galactose metabolism is presented in conjunction with a review of the human galactosemias.

Mammalian Galactose Metabolism

The sequence of metabolic steps by which D-galactose is utilized in mammals was largely elucidated by Leloir (1), Kalckar (2) and their co-workers. Initially, galactose is phosphorylated by a soluble galactokinase and ATP to form galactose 1-phosphate and ADP. Next, the uridyl portion of a uridine diphosphate glucose molecule is transferred to galactose 1-phosphate yielding uridine diphosphate galactose and glucose 1-phosphate. The enzyme catalyzing this reaction, galactose 1-phosphate uridyl transferase, is the second in the three enzyme system depicted in Figure 1. Following the coupling of galactose to a uridine nucleotide, uridine diphosphate glucose is regenerated from uridine diphosphate galactose by an epimerase-catalyzed inversion of the fourth carbon hydroxyl of the galactose moiety. The net result of the cycle is then the conversion of galactose to glucose 1-phosphate which can then be readily

assimilated through the glycolytic pathway. All of these enzymes have been described in a variety of mammalian tissues, such as liver (3-5), red cells (6-8), fibroblasts (9, 10), heart (11), brain and kidney (12-14). The importance of the uridine nucleotide pathway in the metabolism of galactose can be appreciated by considering the hereditary deficiency of either galactokinase or galactose 1-phosphate uridyl transferase. Associated with both conditions is a reduction to about 20% of the normal capacity to oxidize galactose to carbon dioxide (15).

Galactose 1-phosphate can also enter the sugar-nucleotide or Leloir pathway when activated with uridine triphosphate by uridine diphosphate galactose pyrophosphorylase. This reaction, first described in liver by Isselbacher (16, 17) and since described in fibroblasts (18), involves the reversible pyrophosphorolytic cleavage of UTP by galactose 1-phosphate to form UDP-galactose and PPi (Reaction 4, Figure 1). The activity of this enzyme relative to galactose 1-phosphate uridyl transferase varies considerably in different tissues. In human fibroblasts the enzymes are of comparable activity (10, 18) while human liver transferase is about one hundred fold more active than the pyrophosphorylase (19). Evidence that this enzyme is distinct from UDP-glucose pyrophosphorylase (Reaction 5, Figure 1) is at best equivocal (18). Although the synthesis of UDP-glucose or UDP-galactose is reversible via the pyrophosphorylase enzymes, UDP-glucose and UDP-galactose can be hydrolytically

degraded to sugar monophosphates and UMP by a sugar nucleotide pyrophosphotase (Reactions 6, Figure 1). Uridine diphosphate glucose and UDP-galactose are not only intermediates of the Leloir pathway but are precursors to polysaccharide, mucopolysaccharide, uronide conjugates and protein- and lipid-based glycomacromolecules for example.

While galactose is principally metabolized through the Leloir pathway, a number of auxiliary routes have been described. Galactitol, the reductive product of galactose, has been found in the tissues of patients with galactokinase and uridyl transferase deficiencies (20, 21). Rats or chicks fed large amounts of galactose also accumulate galactitol in their tissues (22, 23). The reduction of galactose to galactitol (Reaction 7, Figure 1) can be catalyzed by either aldose reductase (24, 25) or L-hexonate dehydrogenase (26, 27) both of which are found in most mammalian tissues (28). Michaelis constants of 20 mM and 160 mM for aldose reductase and hexonate dehydrogenase respectively reflect the low affinity these enzymes have for galactose (26). While galactitol is not normally found in tissues, it does accumulate when the intracellular galactose concentration approximates the Km of the reductive enzymes. Galactitol accumulation can however be prevented, if the tissues are first treated with tetramethylene glutaric acid, an inhibitor of aldose reductase (29).

The subsequent metabolism of galactitol has not been extensively studied. Lens and liver polyol dehydrogenases,

which oxidize many polyols to keto sugars, do not utilize galactitol as a substrate (30). However, Weinstein, et al. have reported an ATP, NAD^+ and $NADP^+$ dependent oxidation of labeled galactitol to $^{14}CO_2$ by kidney and liver homogenates (31). Since the rate of galactitol oxidation by the kidney was only about 3% the rate of galactose or sorbitol oxidation, oxidative metabolism of galactitol must be considered insignificant. Indeed within twenty-four hours after the intravenous administration of radioactive galactitol to humans, as much as 99% is excreted unchanged in the urine with less than 0.5% of the label found in expired carbon dioxide (31).

Cuatrecasas and Segal (32) have described an enzyme from rat liver which catalyzes the direct oxidation of galactose to galactonic acid (Reaction 8, 9, Figure 1). Although the existence of this enzyme has been disputed (33, 34), the presence of galactonic acid in the urine of normal and galactosemic individuals (35) supports the presence of an in vivo oxidative pathway for galactose. The further metabolism of galactonic acid to D-xylulose through a β-keto galactonate intermediate has also been described (Reaction 10, 11, Figure 1) but the evidence is equivocal (36). Xylulose could then be phosphorylated by liver xylulokinase (37) and subsequently metabolized through the hexose monophosphate shunt (Reaction 12, Figure 1).

In vivo studies with muscle phosphoglucomutase have demonstrated the conversion of galactose 1-phosphate to

galactose 6-phosphate (38) (Reaction 13, Figure 1). Although the mechanism of this transformation is probably the same as for the glucose analogues (39), the reaction proceeds at less than 0.5% of the rate using glucose 1-phosphate as substrate. Sols and Crane (40) and others (41) have shown that brain hexokinase will phosphorylate galactose to galactose 6-phosphate (Reaction 14, Figure 1) but at a much slower rate than glucose phosphorylation. Galactose 6-phosphate, once formed, could become substrate for a liver microsomal dehydrogenase to form 6-phosphogalactonic acid (Reaction 15, 16, Figure 1) (42). Subsequent oxidation of 6-phosphogalactonate to pentose phosphates has not been demonstrated (Reaction 17, 18, Figure 1) (43).

The isomerization of galactose 6-phosphate to tagatose 6-phosphate is known to occur in bacterial systems (Reaction 19, Figure 1) (44). While this isomerization has not been demonstrated in higher organisms, beef brain preparations will rapidly convert tagatose 6-phosphate to triose phosphates, presumably with tagatose 1, 6-diphosphate as the intermediate (Reaction 20, 21, Figure 1) (45).

The Galactosemias

Each of the enzymes of the Leloir pathway are now known to have at least one associated genetic abnormality in man (46). Since the literature on the galactosemias is extensive and excellent reviews are available elsewhere (46, 47) only a brief summary of some clinical, genetic and biochemical aspects of the diseases will be presented here.

Galactokinase Deficiency. The first recognized case of galactokinase deficiency was reported by Gitzelmann in 1965 (48). The only clinical manifestation of the disease is the appearance of cataracts within the first few months of life (49). While a few patients with galactokinase deficiency have been described as having neurological disorders (50, 51), it is not considered a characteristic of the disease (50). The onset of lenticular opacities, which if unchecked can lead to blindness, is prevented or even reversed if galactose is omitted from the diet (52-56). Since the development of cataracts is not apparent for several months, early detection of galactokinase deficiency is dependent on routine screening of blood and urine for galactose. Shortly after the ingestion of galactose as lactose, galactose is found in the urine and moderately high concentrations are found in the blood, about 5 mM (52, 56). Smaller quantities of galactitol (20, 51, 56) and galactonic acid (51) are also excreted in the urine following galactose ingestion.

Galactokinase deficiency is inherited as an autosomal recessive trait (56, 57). Estimates of the occurence of the homozygous condition range from 1 in 46,000 to 1 in 84,000 depending on the population analyzed (57, 58). The heterozygote has about one-half normal levels of galactokinase, and except for the occasional appearance of cataracts are asymptomatic (54, 59, 60).

The loss of galactokinase activity in the tissues of

the homozygote is believed to be complete (51), although uridyl transferase and epimerase activities are normal (20, 50, 52, 56). Despite the lack of this enzyme, galactose can still be slowly oxidized to carbon dioxide (51). Since these patients oxidize C-1 of galactose at a greater rate than C-2 and since galactonic acid is found in their urine, a direct oxidative pathway for galactose would seem to be functioning *in vivo*. Studies on the incidence of galactokinase deficiency in persons developing cataracts before the age of forty have revealed a rate of occurence of about 1 in 50 (61). Cataractogenesis in galactokinase deficiency is believed to be related to galactitol accumulation in the lens (47). *In vitro* studies have shown that galactitol accumulation and lenticular opacities can be prevented if the tissue is first treated with tetramethylene glutaric acid, an inhibitor of aldose reductase (29).

Galactose 1-Phosphate Uridyl Transferase Deficiency. This disorder, also known as classic galactosemia, is the most severe of the galactosemias. It is readily detected within the first few days of birth by poor appetite, weight loss, vomitting and diarrhea. Secondary liver involvement as evidenced by hepatomegaly or jaundice results if galactose is not removed from the diet (62). Cataracts have been reported shortly after birth (63) but generally develop after several months (62). Mental retardation of varying degrees (62, 64, 65) is a common feature of the disease if galactose ingestion is unrestricted. In the extreme,

continued galactose administration can lead to seizures and death. Other symptomology of classic galactosemia is reviewed elsewhere (47, 62).

As in galactokinase deficiency, galactose, galactitol and galactonic acid are found in the urine following the consumption of galactose (35). However, in marked contrast to galactokinase deficiency, classic galactosemics are characteristically hypergalactosemic [blood galactose greater than 20 to 30 mM (66, 67)] and frequently hypoglycemic (62).

The disruption in phosphate ester metabolism originally described by Schwarz (7), is believed to be a result of the accumulation of galactose 1-phosphate in the tissues (7, 68, 69). Classic galactosemics given an oral galactose tolerance test rapidly accumulate galactose 1-phosphate in their erythrocytes (70). Galactose 1-phosphate has also been found at autopsy in liver, kidney, brain, heart, tongue and adrenal gland (71). Another feature in common with galactokinase deficiency is high levels of tissue galactitol (21, 72, 73) which is also believed to be related to cataract formation.

Classic galactosemia is also an autosomal recessively inherited disease (74-76). The most recent estimate of its frequency of occurence is 1 in 45,000 (77). The heterozygote has one-half normal levels of erythrocyte uridyl transferase and is clinically healthy except for occasional minor symptomology on heavy galactose loading (62).

At this point it is useful to classify the variants of

classic galactosemia into two categories based on clinical symptomology. The first variant of classic galactosemia, the Duarte variant, was described by Beutler and co-workers in 1965 (78). The Duarte gene is allelic with the galactosemic gene and is also autosomal recessively transmitted (79-81). Erythrocyte uridyl transferase activity of the Duarte homozygote is about 50% of normal and about 75% for the heterozygote (78). Starch-gel electrophoresis of Duarte homozygote erythrocyte transferase reveals a three-banded pattern (82), whereas the normal gives a single band. The Duarte variant is not associated with any disease state or clinical problem. Another variant exhibiting a three-banded electrophoretic pattern [but with a different distribution of activity amongst the bands] and no clinical symptomology has been recently described by Ng, et al. (83). The homozygote of this variant [The Los Angeles variant] has about 150% the normal activity of erythrocyte transferase and the heterozygote has about 110% of the normal. Mixed genotypes of Duarte, Los Angeles, galactosemic and normal individuals have also been described (83).

Another slow-moving electrophoretic form of transferase, termed the Rennes variant, was reported by Schapira and Kaplan (84). This mutant is characterized by about 7% of normal erythrocyte transferase activity. An unstable transferase [Indiana variant] associated with an incomplete loss of activity has also recently been reported (85). Both the Rennes and Indiana variants show the clinical symptomology of

classic transferase deficiency.

A third mutant, the Negro variant, has no erythrocyte transferase activity but does have about 10% of normal activity in the viscera (86-88). Patients with this disorder are able to metabolize intravenously administered galactose to carbon dioxide as well as normal individuals (15). Interestingly, the Negro variant also suffers the same symptomology as the classic galactosemic (90).

Despite normal levels of galactokinase and UDP-galactose epimerase in the classic galactosemic (9), the loss of transferase activity is complete (91). Immunologic studies have shown that the uridyl transferase gene product is synthesized but it is not enzymatically active (92). As in galactokinase deficiency, classic galactosemics are able to slowly oxidize galactose to carbon dioxide (15). Again, C-1 of galactose is oxidized faster than C-2 (89) and since galactonic acid is present in the urine, a direct oxidative pathway for galactose seems to be operating.

Other mechanisms have been proposed to account for the residual galactose metabolism, notably the synthesis of UDP-galactose from UTP and galactose 1-phosphate via UDP-galactose pyrophosphorylase (16), thus bypassing the uridyl transferase reaction. Since this bypass cannot occur in the galactokinase deficient individual, and since the extent of galactose oxidation to carbon dioxide is similar in both diseases (51), the direct oxidation of galactose is a more likely alternative pathway in classic galactosemia.

The molecular events underlying the complex symptomology of uridyl transferase deficiency are not completely understood. While it seems certain that the accumulation of galactose 1-phosphate is involved, the exact mechanisms are unknown. Studies by Pennington and Prankerd (66), Mayes and Miller (93), Ng (94), Donnell et al. (95) and Wells (96) suggest that galactose 1-phosphate may be involved in a futile cycle of ATP hydrolysis leading to a reduction in cellular energy reserves. Also, since the blood galactose levels encountered in uridyl transferase deficiency are many times higher than in galactokinase deficiency, galactose competition for glucose entry into the cell has been proposed as a factor contributing to the etiology of classic galactosemia (97, 98). Other mechanisms, such as the inhibition of normal enzyme systems by galactose 1-phosphate (99-101), hyperosmolar dehydration of nervous tissue (102), increased fragility of neural lysosomes requisite to the release of acid hydrolases (103) and disruption of phospholipid metabolism (104) have also been suggested.

UDP-Galactose 4'-Epimerase Deficiency. This abnormality, only recently discovered by Gitzelmann (65), has only one known homozygote. The condition was accidentally discovered during routine blood galactose screening. What initially seemed to be elevated blood galactose was later determined to be galactose 1-phosphate. Subsequent analysis of erythrocyte galactokinase and uridyl transferase revealed normal levels of these enzymes. In vitro studies with the

patients erythrocytes showed only insignificant metabolism of ^{14}C-labeled galactose or UDP-galactose to $^{14}CO_2$ but ready conversion of ^{14}C-labeled UDP-glucose to $^{14}CO_2$. Epimerase deficiency was then proved by demonstrating the inability of erythrocytes to convert ^{14}C-labeled UDP-galactose to ^{14}C-labeled UDP-glucose.

Analysis of the red and white blood cells showed no epimerase activity although cultured liver and fibroblast biopsy specimens show normal epimerase levels (106). When galactose tolerance tests were administered, blood galactose was only mildly elevated [approximately 1 mM] whereas erythrocyte galactose 1-phosphate was markedly increased to levels similar to those seen in classic galactosemia. Urinary excretion of galactose and galactitol following milk ingestion was insignigicant although a mild galactosemia was observed following tolerance tests.

A study of the family of the patient revealed individuals with only half normal erythrocyte epimerase levels. When these persons were considered heterozygous for epimerase deficiency, a clear recessive inheritance pattern was seen.

To date [the child is now two years old], no clinical or biochemical pathology has been associated with the deficiency. Since the loss of epimerase activity seems to be restricted to the blood tissues, multiple forms of epimerase coded by more than one gene locus would seem to be indicated. Indeed, multiple forms of mammalian liver and erythrocyte epimerase have been reported (107, 108).

Project Rationale and Objectives

Biochemical studies on the pathogenic mechanisms involved in classic galactosemia have relied on the use of model systems. The newborn chick has been useful in this regard since a debilitating neurologic syndrome, believed similar in nature to the human disease, can be produced by the dietary administration of large amounts of galactose (109). Under these conditions, the rate of galactose influx exceeds the metabolic capacity of the tissues. The result is an accumulation of sugar nucleotide and reductive pathway intermediates, much the same as in uridyl transferase deficiency (23, 96, 104, 110).

Despite the success of the chick as a model, a system more closely allied to man would be desirable. The only mammalian system studied to date has been the rat (111-115). Unfortunately, the rat is relatively resistant to the toxic effects of galactose loading (116) presumably due to an active liver galactose metabolism (117, 118). However, studies by Tygstrup and Keiding (119, 120) have shown that ethanol will greatly increase the susceptibility of rats to galactose toxicity. Ethanol administration increases the hepatic NADH/NAD^+ ratio which is inhibitory to UDP-galactose epimerase (121, 122). Again, an accumulation of the Leloir pathway intermediates results (123). Since the success of this model depends on altering the intracellular redox state and has a secondary effect of trapping uridine phosphates (123) [effects which galactose alone does not produce], this

approximation of the galactosemic state must be regarded with skepticism.

Isolated mammalian organ systems have not been used for the study of galactose toxicity although galactose metabolism in various rat tissues has been investigated. Since the ability to metabolize galactose varies considerably in rat tissues (118, 124), the possibility of using a single organ to mimic the galactosemic state was investigated. Previous work by Fisher and Lindsay with perfused rat hearts estimated that galactose utilization [determined as non-glucose reducing substance] was no more than 5% of glucose utilization (125). Furthermore, Quan-Ma and Wells had shown that rats fed galactose-enriched diets accumulate large amounts of galactitol in cardiac tissue (22). These observations, along with the fact that heart preferentially utilizes ketone bodies and fatty acids to carbohydrate fuels (126-129), suggested that an active galactose metabolism may not occur in the rat myocardium. Since isolated metabolic studies could readily be performed with perfusion systems, a rat heart model for studying the toxic effects of galactose was particularly attractive. Accordingly, studies were undertaken to investigate the mechanisms of galactose metabolism in rat heart. Initial experiments were performed to determine the overall ability of cardiac tissue to dispose of galactose via oxidative or reductive pathways. Subsequent experiments were designed to correlate the kinetic properties of the Leloir sugar nucleotide

enzymes with the observed patterns of galactose metabolism. Throughout these experiments, selected comparisons were made with rat liver and brain.

During the course of these investigations, evidence for an alternate 6-phosphorylative pathway of galactose metabolism in rat heart and brain was obtained. Since such a pathway might further the understanding of the residual galactose oxidation observed in galactosemics, the last portion of this work is concerned with the study of this pathway in heart and brain.

Organization

The body of the thesis has been divided into two sections, each following the format used in most biochemical journals. Portions of both sections have been accepted for publication in Archives of Biochemistry and Biophysics and are currently in press under the title "Studies on Galactose Metabolism in Heart and Brain: The Identification of D-Galactose 6-Phosphate in Brains of Galactose-Intoxicated Chicks and Rat Hearts Perfused with Galactose" by W. Donald L. Musick and William W. Wells. A second manuscript dealing with material from the second section titled "Studies on Galactose 6-Phosphate Metabolism in Rat Heart and Brain" has also been submitted, under the same authorship, to Archives of Biochemistry and Biophysics. An appendix has been included to detail methods and results not adequately described in the text.

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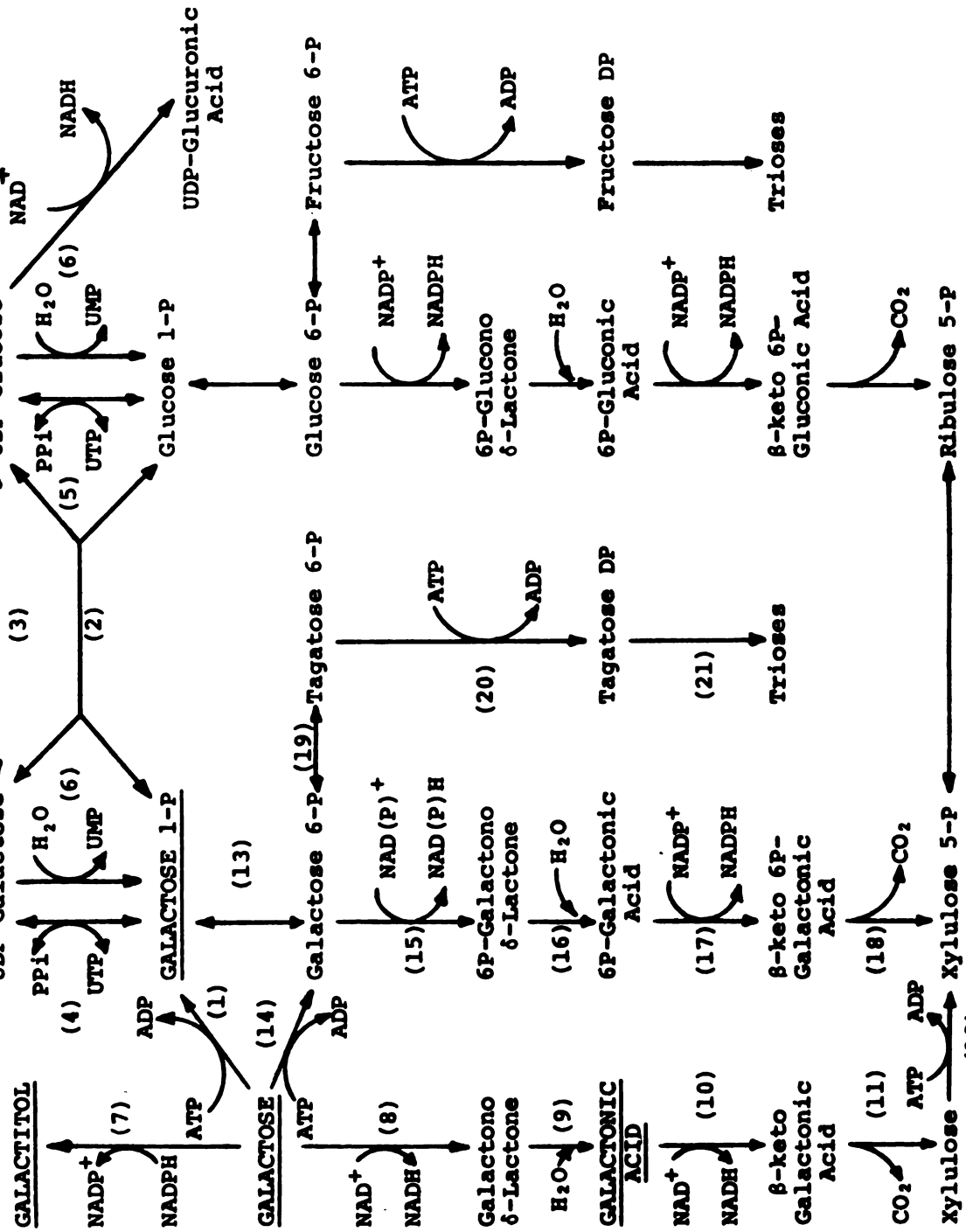
Figure 1. Metabolic Interconversions of D-Galactose.

Underlined metabolites are those known to accumulate in the galactosemias. Numbered reactions are: 1. galactokinase (E.C. 2.7.1.6); 2. galactose 1-phosphate uridyl transferase (E.C. 2.7.7.12); 3. UDP-galactose 4'-epimerase (E.C. 5.1.3.2); 4. UDP-galactose pyrophosphorylase (E.C. 2.7.7.10); 5. UDP-glucose pyrophosphorylase (E.C. 2.7.7.9); 6. UDP-glucose or UDP-galactose pyrophosphotase (E.C. 3.6.1._); 7. aldose reductase (E.C. 1.1.1.21) or L-hexonate dehydrogenase (E.C. 1.1.1.19); 8. galactose dehydrogenase (E.C. 1.1.1.48); 9. galactono δ-lactone lactonase (E.C. 3.1.1._); 10. galactonic acid dehydrogenase (E.C. 1.1.1._); 11. β-keto galactonic acid decarboxylase; 12. xylulokinase (E.C. 2.7.1.17); 13. phosphoglucomutase (E.C. 2.7.5.1); 14. hexokinase (E.C. 2.7.1.1); 15. hexose 6-phosphate dehydrogenase (E.C. 1.1.1._); 16. 6-phosphogalactone δ-lactone lactonase (E.C. 3.1.1._); 17. 6-phosphogalactonic acid dehydrogenase (E.C. 1.1.1._); 18. β-keto 6-phosphogalactonic acid decarboxylase; 19. galactose 6-phosphate isomerase (E.C. 5.3.1._); 20. fructose 6-phosphate kinase (E.C. 2.7.1.11); 21. aldolase (E.C. 4.1.2.13).


GALACTITOL
NADP+
NADPH
(7)
ATP
ADP
(1)
GALACTOSE
(14)
ATP
ADP
GALACTOSE 1-P
(13)
UDP-Galactose
PPi
UTP
(4)
H2O
(6)
UMP
UDP-Glucose
(3)
(2)
PPi
UTP
(5)
H2O
(6)
UMP
Glucose 1-P
NAD+
NADH
UDP-Glucuronic Acid
Galactose 6-P
(19)
Tagatose 6-P
Glucose 6-P
Fructose 6-P
NAD+
NADH
(8)
Galactono δ-Lactone
H2O
(9)
GALACTONIC ACID
NAD(P)+
NAD(P)H
(15)
6P-Galactono δ-Lactone
(16)
H2O
6P-Galactonic Acid
ATP
ADP
(20)
Tagatose DP
(21)
Trioses
NADP+
NADPH
(17)
β-keto 6P-Galactonic Acid
NADP+
NADPH
6P-Gluconono δ-Lactone
H2O
6P-Gluconic Acid
ATP
ADP
Fructose DP
Trioses
NAD+
NADH
(10)
β-keto Galactonic Acid
CO2
(11)
ATP
ADP
Xylulose
(12)
(18)
CO2
Xylulose 5-P
Ribulose 5-P
NADP+
NADPH
β-keto 6P-Gluconic Acid
CO2

CHAPTER I

CHARACTERIZATION OF D-GALACTOSE METABOLISM IN RAT HEART

Abstract

The ability of adult rat heart to metabolize D-galactose via the sugar-nucleotide and reductive pathways was studied. Rat myocardial slices oxidized D-[1-^{14}C] galactose to $^{14}CO_2$ at less than 5% the rate of D-[1-^{14}C] glucose oxidation. Furthermore, heart homogenates poorly converted D-[1-^{14}C] galactose into phosphorylated intermediates under conditions in which 25% of the labeled galactose was converted to metabolites by liver homogenates.

The Leloir pathway enzyme activities of rat heart were found to be present in amounts 8%, 38% and 23% that of rat liver for galactokinase, galactose 1-phosphate uridyl transferase and UDP-galactose 4'-epimerase respectively. The corresponding values for the rat brain enzymes relative to liver were 27%, 11% and 120% respectively. Michaelis constants for the three enzymes were comparable in heart, brain and liver.

The reduction of galactose to galactitol in perfused heart preparations was dependent on the perfusate galactose concentration and appeared to be substrate saturable with a

"Km" of 30mM for galactose. The rate of galactitol production in perfused rat hearts was several times greater than the rate of galactose oxidation to carbon dioxide by heart slices at equivalent perfusion and incubation media galactose concentrations. Increasing the perfusate galactose concentration also had the effect of reducing myocardial ATP and creatine phosphate levels.

Rat heart and brain, but not liver, crude enzyme preparations produced what appeared to be galactose 6-phosphate in addition to galactose 1-phosphate when incubated with high concentrations of D-galactose [30 mM]. Tissue hexokinase activities were measured as a possible explanation for galactose 6-phosphate synthesis.

Introduction

Investigations on the pathogenic factors involved in classic uridyl transferase deficiency have benefited from the availablity of human material. However, the use of experimental model systems has been invaluable in understanding the disease process. The most extensively studied model to date has been the newborn chick (1-12). Due to the low activities of the Leloir pathway enzymes in chick brain [especially of galactose 1-phosphate uridyl transferase] galactose is not appreciably metabolized by brain (3,4). Consequently, when large amounts of galactose are administered in the diet, the influx of galactose exceeds the capacity of brain to metabolize galactose and an accumulation of phosphorylated and reduced metabolites results. (2-4, 8, 11). Concurrently,

a depression in glycolytic- and energy-metabolite levels are observed (2, 6, 11).

Studies by Quan-Ma and Wells have shown that rats fed galactose-enriched diets accumulate large amounts of galactitol [a dead end metabolite (13)] in heart tissue (14). Furthermore, Fisher and Lindsay have estimated that recirculating perfused heart preparations remove galactose from the perfusing medium at no more than 5% the rate of glucose removal (15). These observations suggested that galactose is not actively oxidized by rat myocardium. Since controlled metabolic studies can be readily performed on isolated perfused heart preparations, the utility of rat heart as a model galactosemic tissue was investigated. Accordingly, studies were undertaken to determine the ability of rat heart to convert galactose to intermediates of the Leloir and reductive pathways and to carbon dioxide. The activities and kinetic properties of galactokinase (E.C. 2.7.1.6), galactose 1-phosphate uridyl transferase (E.C. 2.7.7.12) and UDP-galactose 4'-epimerase (E,C, 5.1.3.2) in rat heart are compared to those of rat brain and liver and correlated with the observed patterns of galactose metabolism in heart. During these studies, analysis of the products of the reaction catalyzed by crude galactokinase preparations at high galactose concentrations from either brain or heart, but not liver, revealed the presence of a metabolite with the properties of galactose 6-phosphate. A preliminary study of the effects of galactose on rat heart energy metabolism is also reported.

Materials and Methods

Animals and Materials

Adult male albino rats [300-400 g] of the Holtzman strain were fed a commercial diet and water, ad libitum. D-Galactose, D-glucose, phosphoenolpyruvate, ATP, ADP, [grade I], NAD^+ [grade III], $NADP^+$, UDP-glucose, uridine 5'-diphosphoglucose dehydrogenase [type V] (E.C. 1.1.1.22), pyruvate kinase [type II] (E.C. 2.7.1.40), D-glucose 6-phosphate dehydrogenase [type XI] (E.C. 1.1.1.49), sodium heparin [grade I], and bovine serum albumin [Cohn fraction V] were purchased from Sigma Chemical Company. D-[1-^{14}C] galactose [3 mCi/mmole and 52.9 mCi/mmole], D-[1-^{14}C] glucose [2.95 mCi/mmole], and UDP-[U-^{14}C] galactose [255 mCi/mmole] were products of New England Nuclear Corporation. D-[U-^{14}C] Galactose 1-phosphate [5.0 mCi/mmole] and UDP-[U-^{14}C] glucose [200 mCi/mmole] were obtained through Calatomic and Schwarz Bioresearch, respectively. The purity of each radioisotopic metabolite employed was verified by chromatography in the solvent system appropriate to each enzymatic assay. Hexokinase [10 mg/ml] (E.C. 2.7.1.1), D-glucose 6-phosphate dehydrogenase [5 mg/ml] (E.C. 1.1.1.49) and creatine kinase [lyophilized powder, made 10 mg/ml in water] (E.C. 2.7.3.2) were obtained from Boerhinger-Mannheim. Bacterial alkaline phosphatase (E.C. 3.1.3.1) was a Worthington Biochemical Company product and sodium pentobarbital was from Abbott Laboratories. Triton X-100 was purchased from Research

Products International Corporation. Chromatography was performed on Whatman 3MM paper or J. T. Baker cellulose PEI-F plastic TLC sheets. TLC sheets were developed in an Eastman Chromagram Developing Apparatus. Packard 10-X hyamine hydroxide [1M in methanol] was employed as a carbon dioxide trap in isotope oxidation studies. All other chemicals were reagent grade.

Tissue Preparation for Slice Studies

Animals lightly anaesthetized with diethyl ether were decapitated and whole hearts were excised with as little adipose and vascular tissue as possible. Hearts were thoroughly washed free of blood in ice cold saline, and slices were made with an ice-chilled Stadie-Riggs slicer. Approximately ten slices were routinely obtained from each heart. The slices were temporarily placed in ice cold Krebs-Ringer phosphate buffer (16) which had been previously gassed for one hour with an O_2:CO_2 [95:5] mixture, and were incubated within 30 minutes of the animal's death.

Slice Incubation Procedure

Erlenmeyer flasks [50-ml], fitted with rubber septa and plastic center wells, served as incubation flasks. A half-inch square piece of Whatman 3MM paper was placed in the well to serve as a support for a hyamine hydroxide CO_2 trap. Hyamine hydroxide [200 µl] was applied to the paper immediately prior to the incubation period. Three slices [approximately 60 mg dry weight] were added to flasks containing

5.0 ml of oxygenated Krebs-Ringer phosphate buffer, pH 7.4, and either 3 mM D-[1-^{14}C] galactose [0.5 μCi], 33 μM D-[1-^{14}C] galactose [0.5 μCi] or 3 mM D-[1-^{14}C] glucose [0.5 μCi]. The incubations were begun by adding the slices to the flasks at 37°C in a Dubnoff metabolic incubator. The reaction was stopped at the appropriate time by injecting 1 ml of 3 M trichloroacetic acid through the septum. The flasks were then incubated at 37°C for an additional hour. The radioactivity in the hyamine trap was measured by placing the trap in 10 ml of Bray's solution [5 g 2,5-diphenyl oxazole and 100 g naphthalene in 1 liter p-dioxane] and counting at 75% efficiency in a Beckman CPM-100 scintillation spectrometer. The slices were removed from the incubation medium, dried overnight in a vacuum dessicator, and weighed to the nearest mg. Carbon dioxide evolved was expressed as μmoles or nmoles produced/hr/gram dry weight tissue.

Homogenate Preparation and Labeled Intermediate Studies

Hearts and liver samples [right lobe] were removed from animals sacrificed as previously described. The tissues were rapidly placed in ice cold 0.1 M KCl and washed free of blood. Each specimen [1 g] was homogenized in 4 volumes 0.2 M Tris-HCl, pH 7.3, with ten strokes of a Teflon pestle type Potter-Elvehjem homogenizer at 0-4°C. Four ml of each homogenate was placed in a 50-ml Erlenmeyer flask which contained the following: D-[1-^{14}C] galactose [2 mM; 2μCi], phosphoenolpyruvate [2 mM], pyruvate kinase [approximately 40 units],

ADP [0.5 mM], KF [10 mM], $MgCl_2$ [3 mM], β-mercaptoethanol [1.5 mM], and water to a final volume of 1.0 ml. Each flask was incubated at 37°C for 2 hours in a Dubnoff metabolic incubator. At 30-minute intervals, 0.5 ml of each reaction mixture was withdrawn, placed in a test tube in a boiling water bath for 2 minutes. Tubes were cooled to ice temperature and centrifuged at 30,000 x g for 30 minutes at 4°C in a Sorvall SS-34 rotor, and supernatant fractions were spotted in 30 µl aliquots on a 15-inch Whatman 3MM paper chromatogram and developed ascendingly for 20 hours in ethanol:1M ammonium acetate [pH 7.6], 7:3, v/v. The radioactive compounds were located with a Packard Model 7201 Radiochromatogram scanner, cut out and counted in 10 ml of Bray's solution. Since nucleotide sugars co-migrate with sugar phosphates in this solvent system, the following procedure was utilized to distinguish between the two. To 80 µl of the 2-hour liver supernatant incubation mixture were added 20 µl of 1 M Tris-HCl, pH 10.0, and 10 µl [3.3 units] of bacterial alkaline phosphatase. A reaction containing no phosphatase served as a control. Alkaline phosphatase was inactive toward UDP-galactose and UDP-glucose. The tubes were incubated at 25°C for 1 hour, and a 30-µl aliquot of each reaction mixture was spotted on a chromatogran and developed in ethanol:ammonium acetate, as before. After scanning the regions of each chromatogram corresponding to the radioactive areas of the control reaction were cut out and counted as above.

Enzyme Preparations

Leloir Pathway Enzymes. Rat heart, liver [right lobe] and brain samples were removed from animals sacrificed as described and rinsed in ice cold 0.1 M KCl. One gram of heart or liver was homogenized at top speed for 60 seconds in a Sorvall omnimixer in 5 volumes of a solution containing 10 mM phosphate buffer, pH 6.7, 14 mM β-mercaptoethanol, and 1 mM EDTA at 0-4°C. Heart and liver tissues employed in the UDP-galactose 4'-epimerase assays and all brain tissue were homogenized in a Potter-Elvehjem type homogenizer. Homogenates were centrifuged at 1000 x g for 20 minutes and their supernatants centrifuged at 30,000 x g for 30 minutes in a refrigerated Sorvall RC-2B centrifuge with an SS-34 rotor. The 30,000 x g supernatant fractions were centrifuged at 100,000 x g for one hour at 4°C in a Beckman L-2 ultracentrifuge with a 40-K rotor. These final supernatant fractions were used undiluted in the galactokinase assays; however, liver supernates were diluted 1:3 and 1:2 with homogenization buffer for use in galactose 1-phosphate uridyl transferase and UDP-galactose 4'-epimerase assays, respectively. The enzyme preparations were kept on ice and assayed within three hours of tissue removal. Protein was determined by the method of Lowry et al. (17) using bovine serum albumin as a reference.

Hexokinase. Heart, liver [right lobe] and brain samples were removed from animals sacrificed as described and rinsed in ice cold 0.32 M sucrose containing 1 mM β-mercaptoethanol.

One gram of each tissue was homogenized in 9 volumes of ice cold rinsing media in a Potter-Elvehjem homogenizer. Total hexokinase was determined on aliquots of these homogenates which were diluted 1:5 in rinsing media and made 0.5% (v/v) in Triton X-100 [with vortexing]. Deletion of the Triton X-100 gave overt hexokinase activity (18). The original homogenates were centrifuged at 10,000 x g for 30 minutes at 4°C in a Sorvall RC-2B centrifuge with an SS-34 rotor. The 10,000 x g supernatant fractions were recentrifuged at 100,000 x g for 60 minutes at 4°C in a Beckman L-2 ultracentrifuge with a 40K rotor. These final supernates were used undiluted for the soluble hexokinase assays. All enzyme preparations were kept on ice prior to assaying.

Enzymatic Assay Procedures

Galactokinase. The standard assay procedure employed was a modification of that described by Cuatrecases and Segal (19). The reaction was initiated by adding enzyme and was performed at 37°C in an incubation mixture containing ATP [3 mM], $MgCl_2$ [5 mM], KF [5 mM], β-mercaptoethanol [10 mM], Tris-HCl, pH 7.5 [200 mM], D-[1-^{14}C] galactose [0.33 mM; 0.1 μCi], 30 μl of enzyme preparation [about 0.55 mg liver protein, 0.3 mg heart protein, or 0.15 mg brain protein], and water to a final volume of 0.3 ml. The reaction was stopped at the appropriate time by spotting a 30 μl aliquot of the incubation mixture at the origin of a 15-inch Whatman 3MM paper chromatogram. The chromatogram was

developed ascendingly in ethanol:1M ammonium acetate [pH 7.6], 7:3, v/v, for 20 hours. The chromatogram was scanned, and counted as previously described. The migration of galactose 1-phosphate relative to that of galactose is 0.3 in this solvent system. No product was detectable when either ATP or enzyme preparation was omitted from the reaction mixture. The reaction was linear with time for 10 minutes and linear with respect to protein up to 0.75 mg for all enzyme preparations. The standard incubation time was 6 minutes.

Galactose 1-Phosphate Uridyl Transferase. The assay procedure of Bertoli and Segal (20) was used with minor modification. The reaction mixture contained UDP-glucose [0.25 mM], Tris-HCl, pH 8.4 [200 mM], β-mercaptoethanol [7 mM], D-[U-^{14}C] galactose 1-phosphate [0.375 mM; 0.37 μCi], 20 μl of enzyme preparation [about 0.12 mg liver protein, 0.18 mg heart protein, or 0.1 mg brain protein], and water to a final volume of 0.2 ml. The reaction was initiated by adding enzyme and incubating at 37°C for the appropriate times and terminated by placing the assay tubes in a water bath at 90°C for 90 seconds. The tubes were equilibrated to 25°C and 15 μl [5.0 enzyme units] of bacterial alkaline phosphatase were added. After 20 minutes, 20 μl of the reaction mixture were spotted on a paper chromatogram and developed as described in the galactokinase assay. The substrate, galactose 1-phosphate, is converted to galactose by the alkaline phosphatase treatment, which is easily separated from the product UDP-galactose in the solvent system employed.

No product was detected when UDP-glucose or enzyme preparation was omitted from the reaction mixture. Under the conditions of the assay, both substrates, galactose 1-phosphate and UDP-glucose, and the product, UDP-galactose, were shown to be stable to degradation [data not shown]. The assay was linear with time for the 6-minute incubation period and with protein up to 0.3 mg for all enzyme preparations.

UDP-Galactose 4'-Epimerase. A modification of the method described by Cohn and Segal (21) was employed. The reaction mixture contained NAD^+ [2.5 mM], sodium glycinate, pH 9.0 [200 mM], UDP-[U-^{14}C] galactose [100 µM; 0.036 µCi], 20 µl of enzyme preparation [0.16 mg liver protein, 0.18 mg heart protein, or 0.1 mg brain protein], and water to a final volume of 0,2 ml. The reaction, initiated by the addition of 100,000 x g supernatant, was incubated at 37°C for the appropriate time periods, and terminated by placing the tubes in a water bath at 90°C for 90 seconds. Following equilibration of the tubes at 25°C, 0.5 µmole of NAD^+ were added for the subsequent UDP-glucose dehydrogenase catalyzed reaction initiated by adding 0.01 unit of the enzyme. The incubation period was one hour at 25°C, and the substrate and product were separated by applying 10 µl of the reaction mixture at the origin of a previously water washed plastic TLC sheet impregnated with polyethyleneimine and developed ascendingly with 0.2 M LiCl for 3-1/2 hours.

Since UDP-glucuronic acid migrates slowly, it can be separated from the faster moving UDP-galactose. No product

was formed if either NAD^+ or enzyme preparation were omitted from the reaction. The reaction was linear with time for the 6-minute incubation period and with protein up to 0.25 mg for all enzyme preparations.

Hexokinase. Hexokinase was determined by the method of Knull et al. (22) on 20-40 µl aliquots of enzyme preparations. Each cuvette contained HEPES buffer, pH 7.5 [40 mM], $MgCl_2$ [6.7 mM], thioglycerol [10.2 mM], ATP [6.7 mM], glucose [3.3 mM], $NADP^+$ [0.6 mM], glucose 6-phosphate dehydrogenase [1 µl, 1 U/ml, Sigma] and water to a final volume of 0.5 ml. The reaction was initiated with glucose or ATP [liver only] and followed with time at 340 nm.

Determination of Acid Lability of Phosphate Esters

The reaction products of galactokinase assays performed at 30 mM galactose [2 µCi [1-^{14}C] galactose/assay] were localized as previously described. The products corresponding to the hexose phosphates were eluted descendingly from the chromatogram with distilled water. The eluate was concentrated on a rotary evaporator at room temperature and taken up in water to a final volume of 150 µl. Enzymatic hydrolysis of the products was accomplished by adding 5 µl of alkaline phosphatase to 50 µl of the concentrate and incubating at 25°C for 1 hour. Mild acid hydrolysis was performed by adding sufficient HCl to 50 µl of concentrate to yield a 0.25 M solution. The tubes were heated at 100°C for 1 minute, then cooled to ice temperature. The remaining 50 µl of each

concentrate served as a chromatographic standard. The enzymatic and acid hydrolysates along with their respective untreated aliquots were chromatographed on Whatman 3MM paper and developed ascendingly as described in the galactokinase assay. Radioactivity was localized as described previously.

Heart Perfusions

Rats were sacrificed by an intraperitoneal injection of 30 mg sodium pentobarbital. One hour prior to sacrificing, 5 mg of sodium heparin was injected intraperitoneally to prevent blood clotting. The upper abdominal cavity was opened with a V-shaped incision just below the costal margin. The diaphragm was transected and the peritoneal cavity opened with a longitudinal cut running midline from the diaphragm to the top of the sternum. The pericardial membrane was removed with tweezers and the aorta was cut about 1/2 cm from the heart. All other vasculature was cut off as close to the heart as possible. The heart was rapidly rinsed in ice cold saline and attached via an aortic cannula to a Langendorf-type perfusion apparatus as described by Neely et al. (23) (Figure 2). An initial gravity fed retrograde perfusion [5 minutes] from a reservoir 75 cm above the heart was performed to wash out residual blood from the preparation. Subsequently, the heart was mounted in the perfusion chamber and 60 ml of oxgenated perfusion buffer (23) was recirculated through the heart for 30 minutes. The perfusion buffers in all cases contained 2 mM glucose with additional galactose to concentrations of 2, 5, 10 or 15 mM [the washout buffer

contained no galactose]. The entire system was maintained at 37°C and the perfusion pressure held constant at 40 mm Hg. Following the perfusion period, hearts were freeze-clamped by tongs cooled in liquid nitrogen.

Metabolite Analysis

Frozen hearts were pulverized with a mortar and pestle cooled to dry ice temperature. Powdered tissue samples [100 mg] were extracted with perchloric acid as described by Kozak and Wells (2). The neutralized extracts [20 µl aliquots] were analyzed for ATP spectrophotometrically at 25°C in a cuvette containing Tris-HCl, pH 7.3 [100 mM], $MgCl_2$ [5 mM], D-glucose [0.5 mM], $NADP^+$ [1.6 mM], hexokinase [1 µl, 10 mg/ml], glucose 6-phosphate dehydrogenase [2 µl, 5 mg/ml, Boerhinger] and water to a final volume of 0.25 ml. The reaction was initiated with hexokinase and followed at 340 nm. Creatine phosphate was determined in the same cuvette after the addition of 0.04 µmole ADP and 3 µl of creatine kinase [10 mg/ml].

Somogyi extracts of powdered tissue samples [100 mg] were analyzed for galactitol by gas-liquid chromatography (24).

Results

Oxidation of D-Galactose

Figure 3A indicates that the rate of oxidation of [1-^{14}C] glucose to $^{14}CO_2$ by heart slices is linear over the 2-hour incubation period [4 µmoles/hr/gm dry weight]. When [1-^{14}C] galactose at the same concentration and specific radioactivity

was used in place of glucose, only erratic production of $^{14}CO_2$ was observed at a rate no higher than 0.2 μmoles/hr/gm dry weight [data not shown]. However, when carrier galactose was omitted from the incubation mixture and only isotopic galactose employed, a limited amount of $^{14}CO_2$ was produced linearly with time (Figure 3B, 5 nmoles/hr/gm dry weight).

Galactitol Production in Galactose-Perfused Rat Hearts

In order to determine the physiologic role of the reductive pathway in the disposition of galactose by cardiac tissue, rat hearts were perfused for 30 minutes with media containing 2 mM glucose and either 2, 5, 10, or 15 mM galactose. Figure 4 shows that there is a direct relationship between the perfusate galactose concentration and tissue levels of galactitol. If Figure 4 is regarded as a substrate-velocity plot, a double reciprocal analysis of the data gives a Km for galactose of 30 mM. The maximal rate [V_{max}] at which these preparations synthesize galactitol [at saturating galactose] is found to be 0.5 μmoles/30 min/gm fresh tissue. At a galactose concentration of 3 mM, the rate of galactitol production is about 0.05 μmoles/30 min/gm fresh tissue (Figure 4).

Production of Labeled Leloir Pathway Intermediates

Since galactose was not appreciably oxidized by heart slices, the possibility of a block in its enzymatic conversion

to glycolytic metabolites was investigated. Cell-free extracts of liver and heart were incubated with [1-^{14}C] galactose for varying time periods as described in Materials and Methods. Labeled nucleotide sugars and sugar phosphates were separated from neutral sugars by chromatography of extracts of the incubation mixture, as described. Figure 5A shows a radiochromatogram scan of a liver extract incubated for 2 hours with [1-^{14}C] galactose. Approximately 25% of the total radioactivity was found associated with the slower moving materials. This component was identified as galactose phosphate since alkaline phosphatase treatment of the extract produced only neutral hexose (Figure 5B) which was subsequently determined to be galactose [data not shown]. The radioactivity in the hexose phosphate region of the chromatogram increased over the 2-hour incubation period [data not shown].

Heart extracts incubated for two hours with ^{14}C-galactose were similarly chromatographed (Figure 5C). When the paper chromatograms were scanned at the sensitivity employed for the liver products, no peak of radioactivity was seen in the region of the phosphorylated compounds.

Enzymes of the Leloir Pathway

To further characterize rat myocardial galactose metabolism, the relative activities and kinetic parameters of the Leloir pathway enzymes in heart were compared with those of liver and brain.

Galactokinase. A computer program which calculated Michaelis constants by the double reciprocal and Eadie-Hofstee methods was employed for data analysis (25). The kinetic data for rat heart, brain, and liver galactokinase yielded Km values for galactose of 0.17 mM, 0.17 mM and 0.14 mM, respectively (Table 1; Figures 6A, 6B and 6C). It can be seen that liver exhibits the highest galactokinase activity of the three tissues [roughly 12 times the activity of the heart and 4 times that of the brain].

Galactose 1-Phosphate Uridyl Transferase. The Km values of transferase for galactose 1-phosphate were 0.16, 0.17 and 0.09 mM, and for UDP-glucose were 0.18, 0.23 and 0.14 mM for liver, heart and brain, respectively (Table 1; Figures 7A, 7B, 7C, 7D, 7E and 7F). Liver activity was higher than heart or brain [about 2.5 times that of the heart activity and 9 times that of brain]. The ratio of galactokinase to uridyl transferase varied in all three tissues. The ratio in the heart was about 1:10, whereas liver and brain showed ratios of 1:2 and 1:1, respectively. Inhibition by either UDP-glucose or galactose 1-phosphate (20) was not observed at the substrate concentrations employed.

UDP-Galactose 4'-Epimerase. The computed Km values for UDP-galactose of heart, brain and liver epimerase were 0.14, 0.13 and 0.20 mM, respectively (Table 1; Figures 8A, 8B and 8C). Epimerase activity was observed to be the highest in brain [about 5 times the heart activity and 1.5 times that of liver]. The ratio of transferase to epimerase activities

was different in each tissue; about 6:1 for heart, 1:3 for brain and 3:1 for liver. The enzyme preparations from all three tissues exhibited the same requirement for exogenous NAD^+.

Evidence for Galactose 6-Phosphate in Heart and Brain Incubations

The substrate inhibition previously reported for rat liver galactokinase preparations (19,26) was examined in heart, brain and liver at galactose concentrations ranging from 0.1 mM to 35 mM. In these experiments, the liver enzyme activity was inhibited at galactose levels exceeding 3 mM, reaching maximal inhibition [about 35%] at approximately 20 mM (Figure 9). However, heart and brain "galactokinase" activities increased with increasing substrate concentrations of galactose. Since the solvent system employed to separate the substrate from product does not distinguish between positional isomers of galactose phosphates, it was necessary to further characterize the product(s) of the reaction. Enzyme preparations from heart, brain and liver were incubated for 10 minutes at a galactose concentration of 30 mM. The reaction product was isolated as outlined in the experimental procedure. Figures 10, 11 and 12 show the results of treating the hexose phosphate fraction from each tissue with bacterial alkaline phosphatase and mild acid. Figure 10 indicates that the entire liver hexose phosphate product is labile to mild acid treatment. Since mild acid treatment hydrolyzes aldohexose 1-phosphate

esters but not 6-phosphate esters (27), the only detectable product of the liver galactokinase reaction is presumed to be galactose 1-phosphate. However, the "galactokinase" reaction in the heart and brain incubations (Figures 11 and 12) produced a mixture of acid labile, and acid stable, radioactive galactose phosphate esters.

Hexokinase Activity of Rat Heart, Brain and Liver

Particulate and soluble hexokinase from heart, brain and liver were compared. From Figure 13 it can be seen that brain has the highest total hexokinase activity of the three tissues [about 3 times more than heart and 18 times more than liver]. Since about 80% of brain and 60% of heart total hexokinase is particle bound, the soluble hexokinase activities of heart and brain are comparable. Liver hexokinase on the other hand is nearly 100% soluble but still only 1/4 to 1/3 as active as brain or heart soluble activities.

Effect of Galactose on Rat Heart Energy Reserves

Rat hearts perfused with combined glucose and galactose were analyzed for ATP and creatine phosphate. From Table 2, it can be seen that increasing the perfusate galactose concentration has the effect of reducing the tissue concentration of both ATP and creatine phosphate.

Discussion

Although rat cardiac tissue preferentially utilizes fatty acids and ketone bodies as fuels (28-31), glucose can

serve as the sole energy source (32-34). Glucose oxidation by rat heart slices [4 μmoles/hr/gm dry weight tissue] was found to be less than glucose oxidation by perfused heart preparations (28), although differences in the method of substrate introduction make direct comparisons difficult. When galactose was compared to glucose as an oxidative substrate, the rate of ^{14}C incorporation into carbon dioxide from galactose was less than 5% the rate from glucose. This is in marked contrast to other tissues such as liver and kidney where galactose is oxidized at about 1/3 the rate of glucose (35). Since the liver and kidneys actively metabolize galactose (35) and comprise a much larger total tissue mass than the heart, the heart probably contributes little to the overall metabolism of galactose in the rat.

Galactitol synthesis in rat heart was studied in isolated perfused preparations. The tissue concentration of galactitol was found to be directly related to the perfusate galactose concentration and exhibited a "Km" of about 30 mM for galactose. Since mammalian heart is known to have aldose reductase and L-hexonate dehydrogenase activities (36), galactitol synthesis in rat heart is likely a result of both enzymes. Furthermore, since the Michaelis constants for mammalian aldose reductase and hexonate dehydrogenase are 20 mM and 160 mM respectively (37), galactitol production in the perfused heart, over the substrate range examined, is probably due to aldose reductase.

If the dry weight of rat heart is taken as 20% of the

fresh weight, galactose oxidation to carbon dioxide and reduction to galactitol can be roughly compared [the average fresh weight of a rat heart is about 1 gm; see tissue slice preparation in Materials and Methods]. Galactitol synthesis, at 3 mM galactose, is about 0.1 μmoles/hr/gm fresh weight (Figure 4) while galactose oxidation at the same concentration is calculated to be no more than 0.04 μmoles/hr/gm fresh weight tissue. Since the maximal rate of galactose oxidation is likely achieved by 2 mM galactose (35) and the maximal rate of galactitol synthesis is calculated to be about 1 μmole/hr/gm fresh tissue, at higher concentrations of galactose [such as encountered in the classic galactosemic] reduction of galactose probably predominates over oxidation in rat heart. Indeed, Quan-Ma and Wells found that galactitol accumulates to the highest levels in heart and lens when rats are fed galactose-enriched diets (14).

The low rate of galactose oxidation in rat heart slices correlated well with the poor ability of heart homogenates to synthesize phosphorylated intermediates. Liver homogenates on the other hand converted substantial amounts of the labeled substrate to galactose-phosphate in the equivalent time. These observations suggested that galactose phosphorylation was the rate-limiting step in myocardial galactose utilization. This conclusion is supported by the observation that heart galactokinase activity is only obout 10% of heart uridyl transferase activity. In fact, galactokinase activity was the lowest in heart tissue. The Michaelis constants for

galactose in all of the galactokinase preparations (Table 1) agree closely with the literature values of 0.15 mM and 0.14 mM (19, 26) for the adult rat liver enzymes.

Heart uridyl transferase activity was intermediate to that of liver and brain (Table 1). While heart transferase was about 38% as active as the liver enzyme, brain had only 11% of the liver activity. The Km values for galactose 1-phosphate and UDP-glucose in all three tissues (Table 1) were comparable to those previously reported for the rat liver enzyme [0.14 mM for galactose 1-phosphate and 0.16 mM for UDP-glucose] (20).

Cohn and Segal (21) and Weinstein et al. (38) have reported a Km for UDP-galactose of 0.15 mM and 0.17 mM for rat liver and rat intestinal UDP-galactose 4'-epimerase, respectively. Our computed constants from heart, brain, and liver enzyme preparations agree closely, with values of 0.20 mM, 0.13 mM, and 0.14 mM, respectively. The epimerase activity of brain is higher than either brain kinase or transferase.

Although rat heart, like brain (39), does not appreciably metabolize galactose, the underlying cause in each case seems to be different. In the rat brain, galactose 1-phosphate uridyl transferase is lower in activity than galactokinase while epimerase activity is greater than either of the others. It would therefore appear that uridyl transferase is the rate-linking step in rat brain galactose metabolism. This has also been suggested to be the case in chick brain (3). Since galactokinase activity is rate-limiting in

rat heart, the heart appears to be a poor model for uridyl transferase deficiency. However, since the reduction in adenine nucleotide and creatine phosphate levels observed in the chick model (2, 11) and in the classic galactosemic (40) is also seen in galactose-perfused rat heart, heart may be useful in studying the effects of galactose-inhibited glucose transport (6, 41, 42) on cellular energy reserves. Furthermore, heart, unlike brain, is capable of maintaining normal ATP and creatine phosphate in the absence of glucose when fatty acids or ketone bodies are provided (43). Hence, this tissue should be relatively useful in evaluating the consequences of the accumulation of various galactose metabolites on the vital functions of the cell separated from the inhibitory effects of galactose on glucose transport.

The substrate inhibition of rat liver preparations reported by Cuatrecasas et al. (19) and Walker et al. (26) was also observed with our liver enzyme preparations at galactose levels exceeding 3 mM. However, we were unable to demonstrate this effect with either heart or brain galactokinase, due to an apparent galactose 6-phosphorylating activity at higher galactose concentrations. In 1962, Inouye et al. reported that galactose 6-phosphate was produced by erythrocytes of galactosemia patients incubated with galactose. These authors suggested that galactose 6-phosphate might be formed from galactose 1-phosphate via phosphoglucomutase or possibly by the action of a hexokinase on galactose. In our system, the former possibility would seem

unlikely since galactose 6-phosphate was not observed in liver preparations and rat liver phosphoglucomutase activity is 2 times higher than heart and 6 times higher than brain activity (45). On the other hand, soluble hexokinase activities of brain and heart are 3 to 4 times higher than that of liver. Indeed, Sols and Crane (46) and others (47) have shown that brain hexokinase will phosphorylate galactose in the 6-position under conditions of high substrate concentration (10 mM).

The present study provides evidence that at galactose levels observed in severe cases of uridyl transferase deficiency (40, 48), phosphorylated galactose metabolites other than galactose 1-phosphate may have been overlooked. The further characterization of galactose 6-phosphate and its subsequent metabolism are subjects of the following chapter.

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Table 1. Activities and Kinetic Parameters of Rat Heart, Liver and Brain Leloir Pathway Enzymes.

Tissue		Galactokinase	Galactose 1-P Uridyl Transferase		UDP-Galactose 4'-Epimerase
	Substrate	Galactose	Gal 1-P	UDP-Glu	UDP-Gal
Liver	Km, mM	0.14	0.16	0.18	0.20
	Activity*	5.60±0.127 (3)	10.90±0.628 (6)		2.94±0.179 (3)
	Substrate	Galactose	Gal 1-P	UDP-Glu	UDP-Gal
Heart	Km, mM	0.17	0.17	0.23	0.14
	Activity*	0.443±0.033 (3)	4.13±0.343 (9)		0.69±0.036 (3)
	Substrate	Galactose	Gal 1-P	UDP-Glu	UDP-Gal
Brain	Km, mM	0.17	0.09	0.14	0.13
	Activity*	1.51±0.058 (4)	1.24±0.054 (4)		3.57±0.109 (4)

*Activity is expressed as nmoles product formed/min/mg protein ± S. E. M. Bracketed values refer to the number of determinations. Assay conditions are described in Materials and Methods.

Table 2. Effect of Perfusate Galactose Concentration on Rat Heart Energy Reserves.

Perfusate Galactose Concentration (mM)	ATP	Creatine Phosphate
	µmoles/gm tissue	
2	2.72±0.43	2.92±0.68
5	2.05±0.36	2.30±0.62
10	2.16±0.68	1.87±0.80
15	1.95±0.52	2.06±0.02

Values represent duplicate determinations on 4 hearts ± S. D.

Figure 2. Langendorf-type rat heart perfusion apparatus.

Components are as labeled: (A) constant pressure perfusion pump; (B) perfusate reservoir and oxygenation chamber; (C) gas hydration chamber; (D) aortic bubble trap with attached mercury manometer; (E) aortic cannula and mount; (F) gravity-fed washout reservoir; (G) constant temperature circulating water bath.

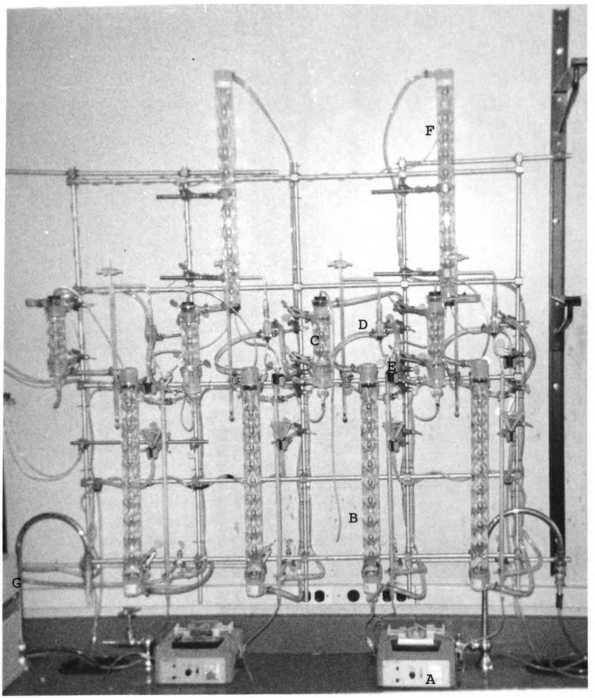

F
D
C
E
B
G
A

Figure 3. Oxidation of D-[1-^{14}C] glucose (A) and D-[1-^{14}C] galactose (B) to $^{14}CO_2$ by rat heart slices.

Three slices [approximately 60 mg, dry weight] of rat heart were incubated in 50 ml Erlenmeyer flasks containing 5.0 ml Krebs-Ringer phosphate buffer, pH 7.4, and either 3 mM D-[1-^{14}C] glucose [0.5 µCi] or 33 µM D-[1-^{14}C] galactose [0.5 µCi] at 37°C. CO_2 released was trapped as described in the Materials and Methods section. Each point represents the mean ± standard deviation of 4 experiments (A) or 6 experiments (B).

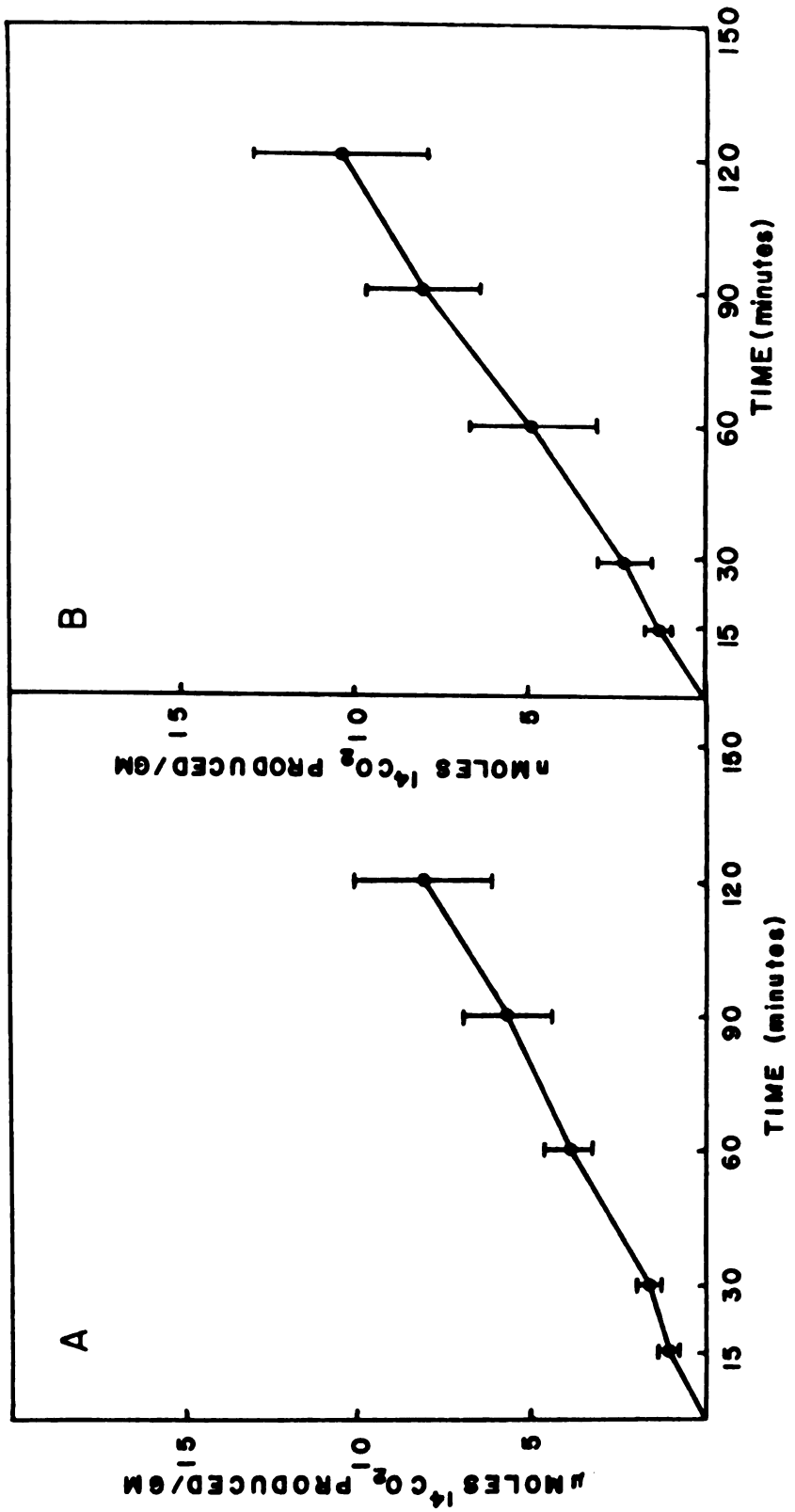

A
B
μMOLES $^{14}CO_2$ PRODUCED/GM
μMOLES $^{14}CO_2$ PRODUCED/GM
15
0
15
5
0
5
15
30
60
90
120
150
TIME (minutes)
TIME (minutes)

Figure 4. The effect of perfusate galactose concentration on perfused rat heart galactitol levels.

Rat hearts were perfused for 30 minutes in a Langendorf-type perfusion apparatus with a perfusion medium containing 2 mM glucose and either 2, 5, 10 or 15 mM galactose. Following the perfusion period, hearts were freeze clamped with liquid nitrogen cooled tongs for metabolite analysis [see Materials and Methods]. Values represent the mean ± S. D. for 4 hearts.

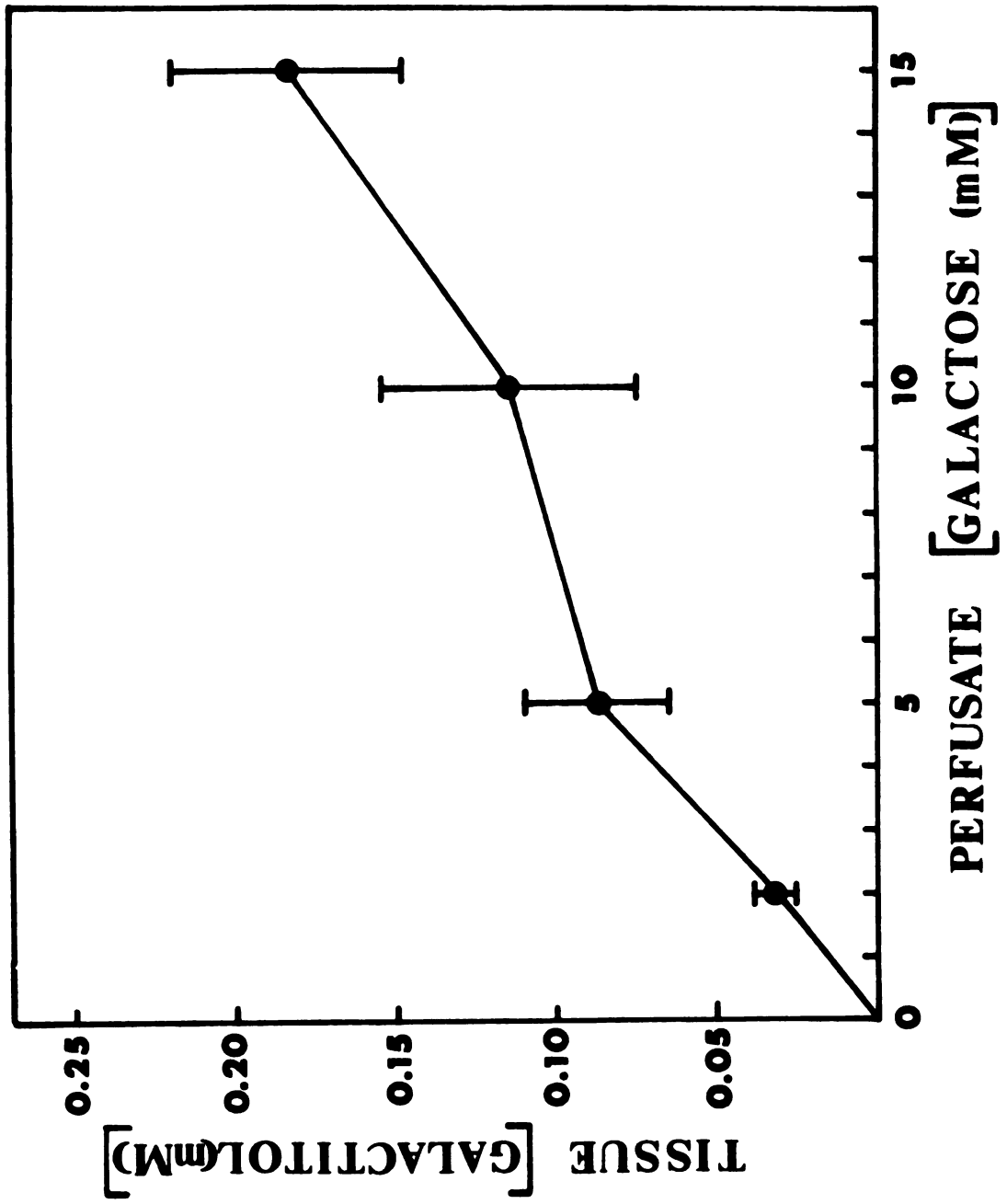

TISSUE [GALACTITOL (mM)]
0.25
0.20
0.15
0.10
0.05
0
5
10
15
PERFUSATE [GALACTOSE (mM)]

Figure 5. Production of labeled Leloir pathway intermediates by rat liver and heart cell free homogenates.

(A) A radiochromatogram scan of paper chromatographed liver homogenate reaction mixtures incubated at 37°C for 2 hours in 50 ml Erlenmeyer flasks containing D-[1-^{14}C] galactose [2 mM, 2 μCi], phosphoenolpyruvate [2 mM], pyruvate kinase [about 40 U], ADP [0.5 mM], KF [10 mM], $MgCl_2$ [3 mM], and β-mercaptoethanol [1.5 mM]. For further details, see the Materials and Methods section.
(B) A repeat scan of the same liver homogenate chromatogram after treatment with alkaline phosphatase.
(C) A similar scan of heart homogenate reaction mixtures incubated as in (A). Vertical arrows denote the regions of the chromatogram corresponding to hexose and hexose monophosphates.

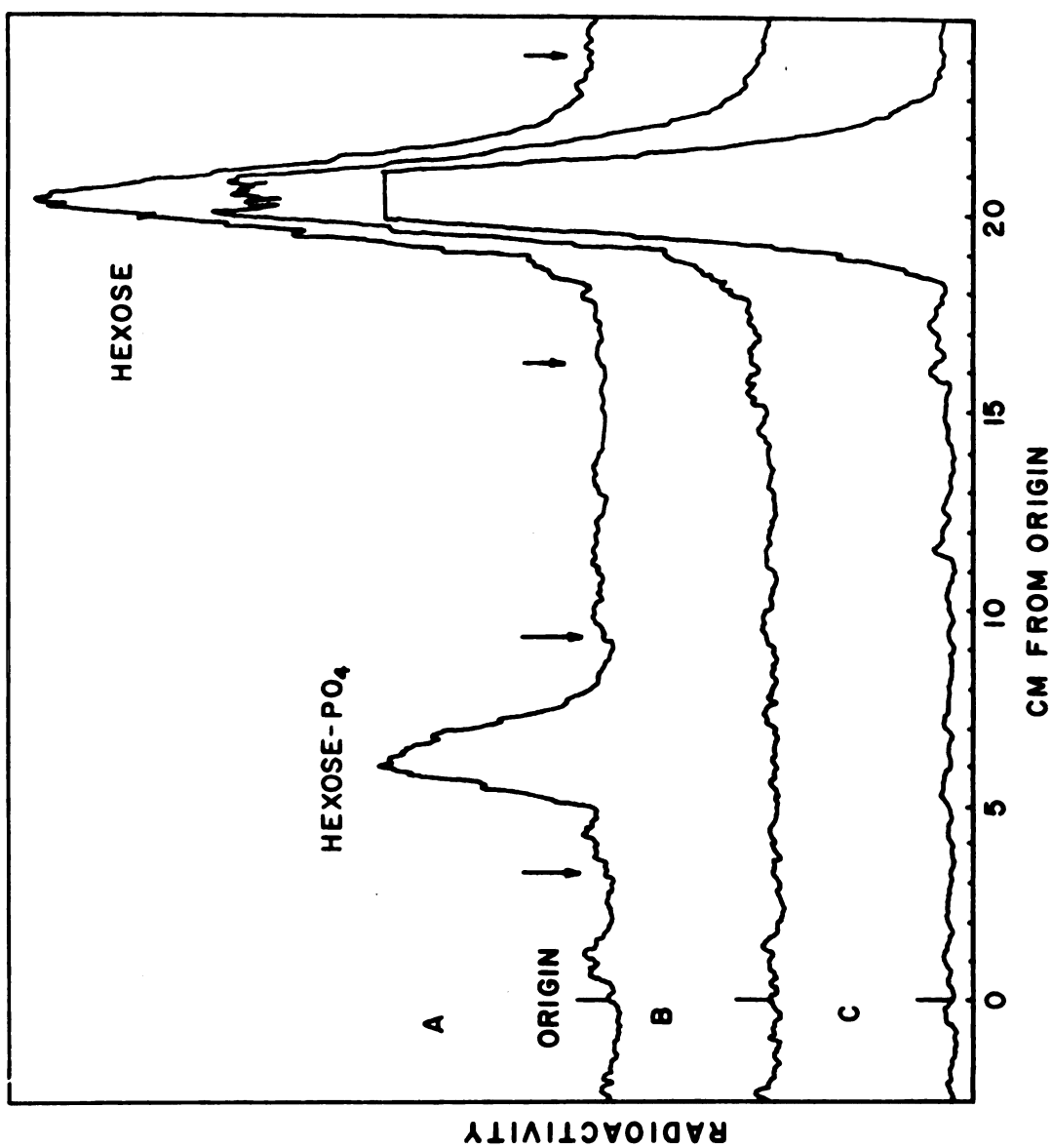

HEXOSE
HEXOSE-PO4
ORIGIN
A
B
C
RADIOACTIVITY
0
5
10
15
20
CM FROM ORIGIN

Figure 6. Reciprocal plots of galactokinase activity in rat liver (A), heart (B), and brain (C) with varying galactose concentrations.

Assays were performed as described in the Materials and Methods section. Each assay tube contained ATP [3 mM], $MgCl_2$ [5 mM], KF [5 mM], β-mercaptoethanol [10 mM], Tris-HCl, pH 7.5 [200 mM], D-[1-^{14}C] galactose at varying concentrations, 30 µl of enzyme preparation and water to a final volume of 0.3 ml. Best fit straight lines were determined by computer. Velocities are expressed as nmoles product/min/mg protein.

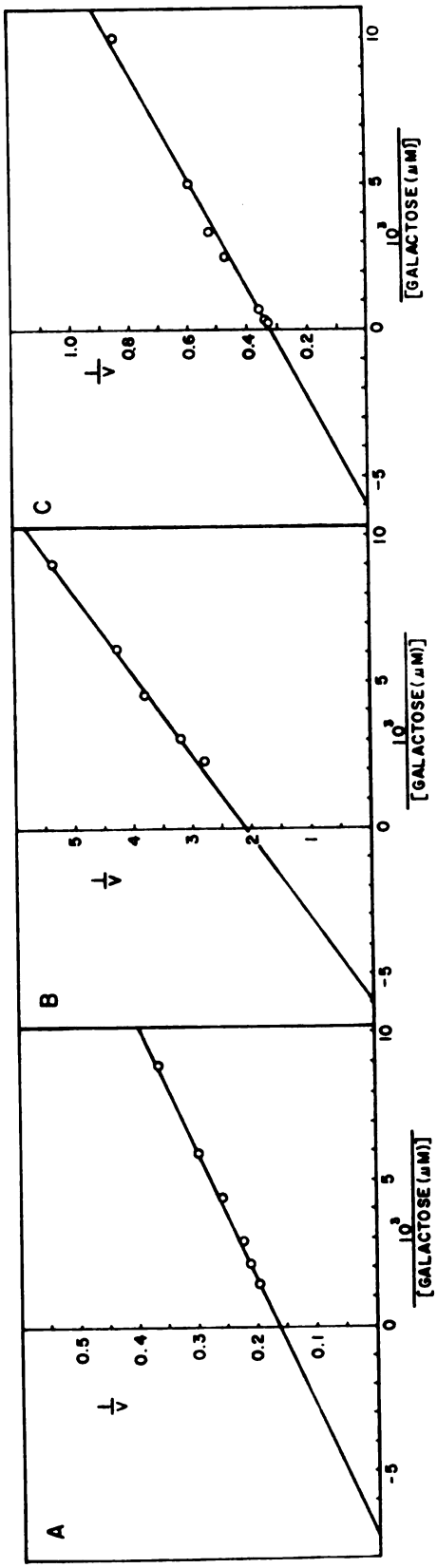

A
B
C
1/v
[GALACTOSE (μM)]
10³

Figure 7. Reciprocal plots for rat liver, heart, and brain galactose 1-phosphate uridyl transferase activity with galactose 1-phosphate and UDP-glucose as the varied substrates.

Assays were performed as in the Materials and Methods section. Figures A and D refer to liver enzyme preparations, B and E to heart, and C and F to brain. Each assay tube contained Tris-HCl, pH 8.4 [200 mM], β-mercaptoethanol [7 mM], UDP-glucose [0.25 mM], with varying D-[U-^{14}C] galactose 1-phosphate concentrations or varying UDP-glucose concentrations with D-[U-^{14}C] galactose 1-phosphate [0.375 mM; 0.37 μCi], 20 μl of enzyme preparation and water to a final volume of 0.2 ml. Velocities are expressed as nmoles product/min/mg protein. Best fit straight lines were obtained by computer analysis.

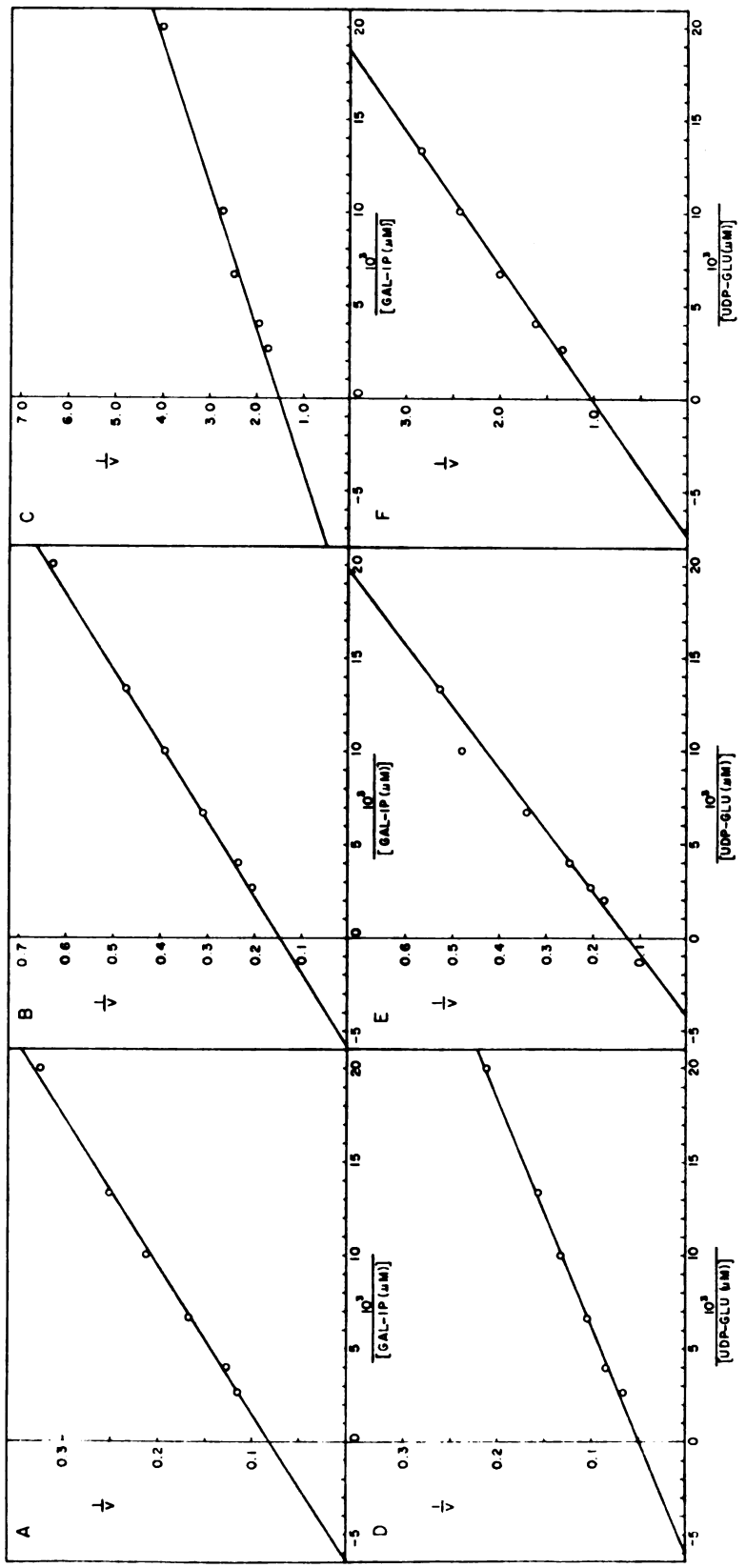

A
B
C
D
E
F
1/v
[GAL-1P (μM)]
[UDP-GLU (μM)]

Figure 8. Reciprocal plots for rat liver (A), heart (B), and brain (C) UDP-galactose 4'-epimerase activity with varying UDP-galactose concentrations.

Assay procedures are described in the Materials and Methods section. Each assay tube contained NAD^+ [2.5 mM], sodium glycinate, pH 9.0 [200 mM], UDP-[U-^{14}C] galactose at varying concentrations 20 µl of enzyme preparation, and water to a final volume of 0.2 ml. Velocities are expressed as nmoles product/min/mg protein. Best fit straight lines were obtained by computer analysis.

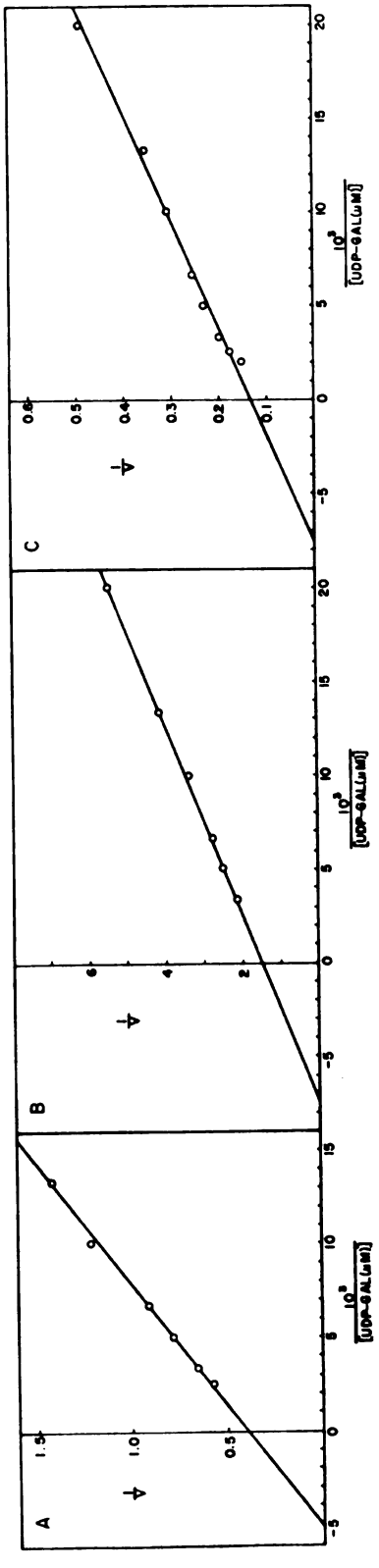

A
B
C
1/v
$\frac{10^3}{[UDP\text{-}GAL(\mu M)]}$

Figure 9. The effect of elevated galactose concentrations on rat liver, heart, and brain galactokinase activities.

A substrate-velocity plot for liver, heart, and brain galactokinase activities with varying galactose concentrations. Assay conditions were those of Figure 6 with a final volume of 0.1 ml. The assays were performed as described in the Materials and Methods section.

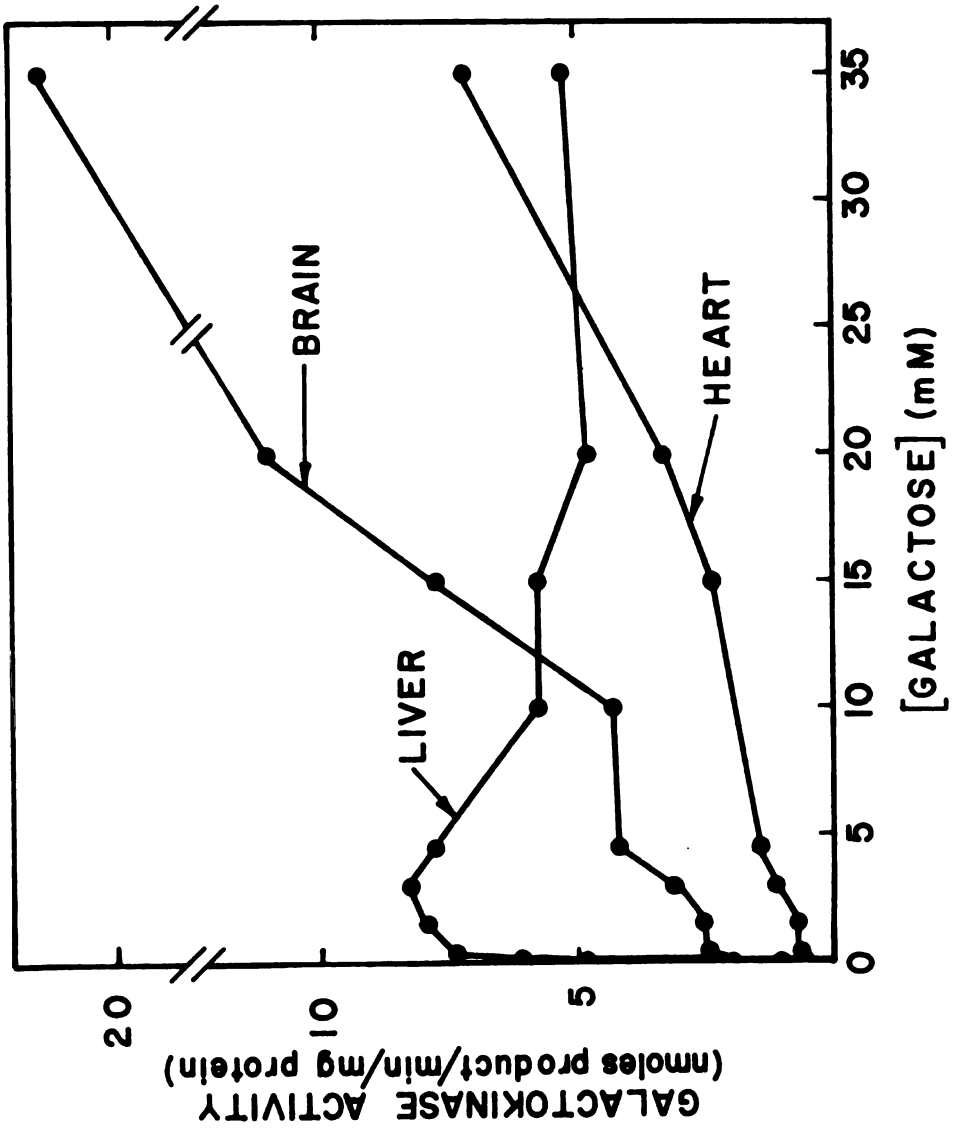

GALACTOKINASE ACTIVITY
(nmoles product/min/mg protein)
20
10
5
0
5
10
15
20
25
30
35
[GALACTOSE] (mM)
BRAIN
HEART
LIVER

Figure 10. Radiochromatography of the products of liver galactokinase reactions performed at 30 mM galactose and the effect of alkaline phosphatase and mild acid hydrolysis on the isolated products.

Galactokinase assays for liver were performed at 30 mM D-galactose [2 μCi] as outlined in Figure 6, with a final volume of 0.1 ml. The radioactive products were isolated by paper chromatography, treated as shown on the Figure and rechromatographed. Details of chromatography, enzymatic, and acid hydrolyses are described in the Materials and Methods section. The vertical arrows denote the regions of the chromatogram corresponding to hexose and hexose monophosphates.

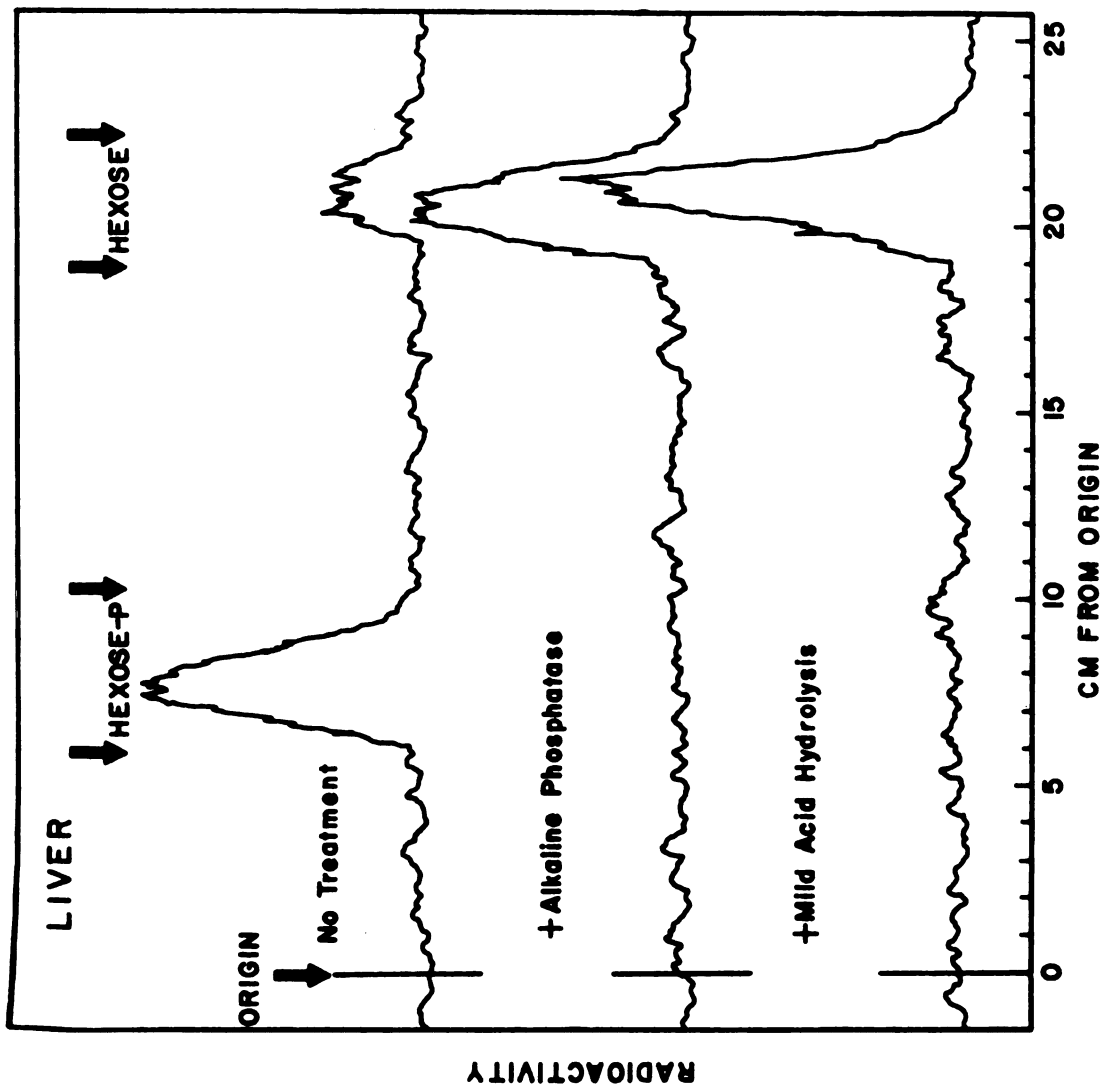

LIVER
HEXOSE-P
HEXOSE
ORIGIN
No Treatment
+Alkaline Phosphatase
+Mild Acid Hydrolysis
RADIOACTIVITY
0
5
10
15
20
25
CM FROM ORIGIN

Figure 11. Radiochromatography of the products of heart galactokinase reactions performed at 30 mM galactose and the effect of alkaline phosphatase and mild acid hydrolysis on the isolated products.

Galactokinase assays for heart were performed at 30 mM D-galactose [2 μCi] as outlined in Figure 6, with a final volume of 0.1 ml. The radioactive products were isolated by paper chromatography, treated as shown on the Figure and rechromatographed. Details of chromatography, enzymatic, and acid hydrolyses are described in the Materials and Methods section. The vertical arrows denote the regions of the chromatogram corresponding to hexose and hexose monophosphates.

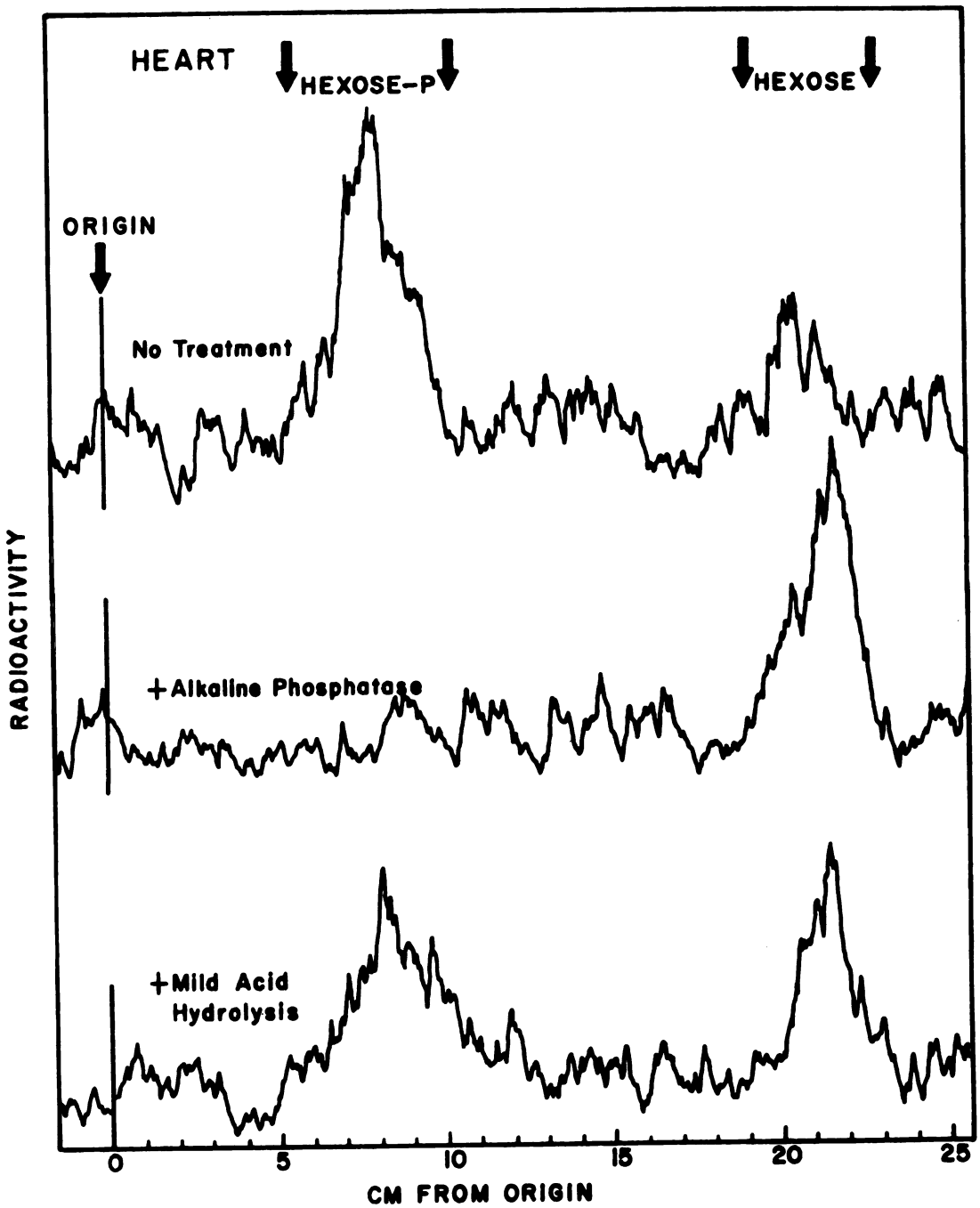

HEART
HEXOSE-P
HEXOSE
ORIGIN
No Treatment
+Alkaline Phosphatase
+Mild Acid Hydrolysis
RADIOACTIVITY
0
5
10
15
20
25
CM FROM ORIGIN

Figure 12. Radiochromatography of the products of brain galactokinase reactions performed at 30 mM galactose and the effect of alkaline phosphatase and mild acid hydrolysis on the isolated products.

Galactokinase assays for brain were performed at 30 mM D-galactose [2 μCi] as outlined in Figure 6, with a final volume of 0.1 ml. The radioactive products were isolated by paper chromatography, treated as shown on the Figure and rechromatographed. Details of chromatography, enzymatic, and acid hydrolyses are described in the Materials and Methods section. The vertical arrows denote the regions of the chromatogram corresponding to hexose and hexose monophosphates.

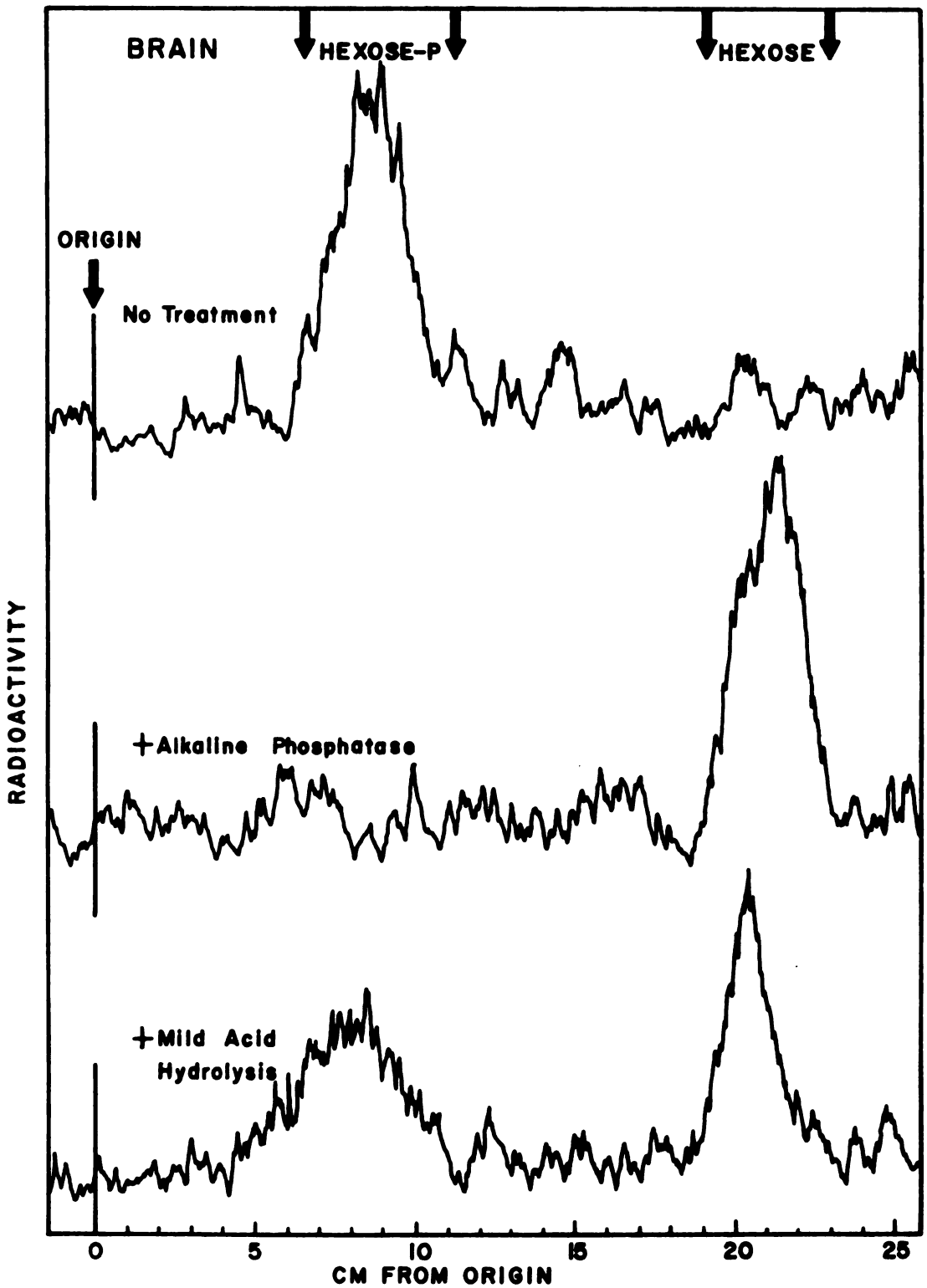
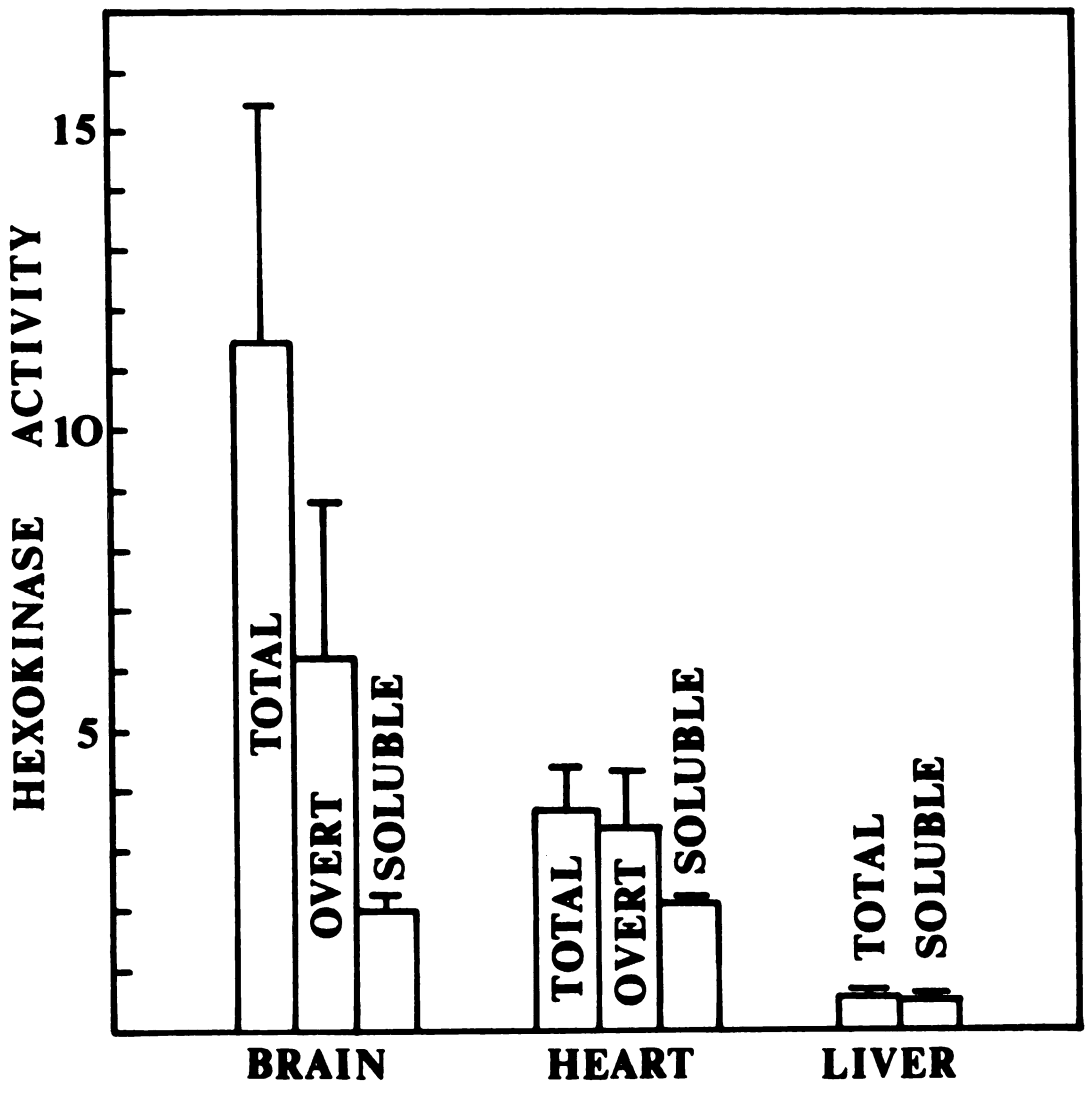

BRAIN
HEXOSE-P
HEXOSE
ORIGIN
No Treatment
+Alkaline Phosphatase
+Mild Acid Hydrolysis
RADIOACTIVITY
0
5
10
15
20
25
CM FROM ORIGIN

Figure 13. Hexokinase activities and distributions in rat brain, heart and liver.

Activity is measured in μmoles glucose phosphorylated/min/gm fresh weight tissue. Values are the averages of triplicate determinations on 2 animals ± S. D.


HEXOKINASE ACTIVITY
15
10
5
TOTAL
OVERT
SOLUBLE
TOTAL
OVERT
SOLUBLE
TOTAL
SOLUBLE
BRAIN
HEART
LIVER

CHAPTER II

IDENTIFICATION AND STUDIES ON THE METABOLISM OF GALACTOSE 6-PHOSPHATE IN HEART AND BRAIN

Abstract

Galactose 6-phosphate was identified in the brains of galactose-intoxicated chicks and rat hearts perfused with galactose by spectrophotometry and analysis by combined gas-liquid chromatography-mass spectrometry. Tissue concentrations of galactose 6-phosphate were compared to selected glycolytic and galactose metabolites. While galactose 6-phosphate levels were comparable to the levels of certain glycolytic metabolites, galactose 6-phosphate only comprised about 5% of the total galactose phosphate.

The subcellular distribution of $NADP^+$ and NAD^+-dependent glucose 6-phosphate and galactose 6-phosphate dehydrogenases were studied in rat liver, heart, brain, and chick brain. Only liver particulate fractions oxidized glucose 6-phosphate and galactose 6-phosphate with $NADP^+$ or NAD^+ as cofactor. While all of the tissues examined had $NADP^+$-dependent glucose 6-phosphate dehydrogenase activity, only rat brain soluble fractions had $NADP^+$-dependent galactose 6-phosphate dehydrogenase activity. Rat liver microsomal and rat brain

soluble galactose 6-phosphate dehydrogenase activities were kinetically different although their reaction products were both 6-phosphogalactonate. Rat brain subcellular fractions did not oxidize 6-phosphogalactonate with either $NADP^+$ or NAD^+ cofactors but phosphatase activities hydrolyzing 6-phosphogalactonate, galactose 6-phosphate and galactose 1-phosphate were found in crude brain homogenates.

The effects of galactose 6-phosphate and 6-phosphogalactonate on several glycolytic and hexose monophosphate shunt enzymes were investigated. 6-Phosphogalactonate was found to be a competitive inhibitor of rat brain 6-phosphogluconate dehydrogenase.

Introduction

Evidence was presented in the first chapter for the synthesis of galactose 6-phosphate at elevated galactose concentrations in rat brain and heart. In order to determine whether galactose 6-phosphate can be synthesized *in vivo*, rat hearts were perfused with high concentrations of galactose. Since rats cannot be made hypergalactosemic by dietary administration of galactose (1), the synthesis of galactose 6-phosphate was not studied in rat brain. Instead, chick brain was studied, since brain and plasma galactose concentrations as high as 25 mM are achieved by feeding galactose-enriched diets (2, 3).

The metabolic utilization of D-galactose in mammals proceeds primarily through the Leloir uridine nucleotide pathway (4, 5). In human galactokinase or uridyl transferase

deficiency where portions of this pathway are blocked, galactose can still be slowly oxidized to carbon dioxide (6-8). A variety of mechanisms have been proposed to account for this residual metabolism such as the direct oxidation of galactose (9, 10), formation and oxidation of galactose 6-phosphate (11, 12), and the synthesis of UDP-galactose from galactose 1-phosphate and UTP (13, 14). The urinary excretion of galactonic acid and the higher rate of galactose C-1 oxidation than C-2 in kinase (8), and transferase (15, 16) deficient patients are consistent with the first two mechanisms. In order to determine whether respiratory carbon dioxide could arise from galactose 6-phosphate, further studies on its metabolism in rat brain and heart relative to liver were undertaken.

Of the three known disorders of the Leloir pathway (17), mental retardation is associated only with uridyl transferase deficiency (18). Since galactose 1-phosphate accumulates in the tissues of these patients (19), attention has been largely focused on galactose 1-phosphate as a toxic metabolite (20-24). This report also describes the effect of galactose 6-phosphate and its oxidative product, 6-phosphogalactonate, on several key glycolytic and hexose monophosphate shunt enzymes.

Materials and Methods

Animals and Materials

Adult male or female albino rats [300-400 gm] of the Holtzman strain were fed a commercial diet and water *ad libitum*. Day-old male white Leghorn chicks were generously profided by MacPherson Hatchery, Ionia, Michigan, and Rainbow Trails Hatchery of St. Louis, Michigan, and kept in brooders at 32°C. For inducing neurotoxicity, chicks were fed *ad libitum* the basal 2 diet of Rutter *et al*. (25), 50% of which contained D-galactose substituted for an equal amount of cerelose [D-glucose monohydrate]. Control chicks were fed the basal 2 diet.

D-Galactose, barium D-galactose 1-phosphate, barium D-galactose 6-phosphate [grade III], disodium D-glucose 1-phosphate, dipotassium D-glucose 6-phosphate, barium D-fructose 6-phosphate [type IV], trimonocyclohexylammonium 6-phospho-D-gluconic acid, NAD^+ [grade III], $NADP^+$, NADH [grade III], ATP, AMP [type II], bacterial alkaline phosphatase [type III-S] (E.C. 3.1.3.1), 6-phospho-D-gluconic acid dehydrogenase [type IV] (E.C. 1.1.1.44), D-glucose 6-phosphate dehydrogenase [type XI] (E.C. 1.1.1.49), D-fructose 6-phosphate kinase [type I] (E.C. 2.7.1.11), D-glucose 6-phosphate isomerase [grade III] (E.C. 5.3.1.9), sodium heparin, and bovine serum albumin [Cohn fraction V] were purchased from Sigma Chemical Company. Other chemicals were reagent grade. Triose phosphate isomerase (E.C. 5.3.1.1), α-glycerol phosphate dehydrogenase (E.C. 1.1.1.8), aldolase (E.C. 4.1.2.13), and β-galactose

dehydrogenase (E.C. 1.1.1.48) were Boerhinger-Mannheim products. Trimethylchlorosilane [TMCS] and N,O,bis-[trimethylsilyl]-acetamide [BSA] were purchased from the Pierce Chemical Company. Triton X-100, bromine, D-glucose, D-galactono-γ-lactone, D-glucono-δ-lactone, and alkaline phosphatase were purchased from Research Products International Corporation, Fisher Scientific Company, Mallinckrodt Chemical Works, Pfanstiehl Laboratories, The California Foundation for Biochemical Research, and the Worthington Biochemical Company, respectively. Barium salts of the sugar phosphates were exchanged with K^+ prior to use.

Tissue Galactose 6-Phosphate

Animal Treatment. Severely intoxicated chicks [52 hours] were decapitated directly into liquid nitrogen. The frozen brains were removed from the skulls with a chisel and pulverized at dry ice temperature in a cold room at 4°C. The powdered tissue was stored in a Revco freezer at -80°C until appropriate assays were performed.

Rats were injected intraperitoneally with 5 mg sodium heparin 1 hour before excision of hearts and were killed by cervical dislocation. Hearts were rapidly removed and washed in ice cold 0.15 M sodium chloride and attached via an aortic cannula to a Langendorf-type perfusion apparatus as described by Neely et al. (26). An initial retrograde perfusion [5 minutes] via the aorta from a reservoir 70 cm above the heart was performed to wash out residual blood from the preparation. Subsequently, a 60 ml recirculating volume of the oxygenated

perfusion buffer described by Neely et al. (26) containing either 30 mM D-galactose or 2.5 mM D-glucose [controls] as the sole energy source was initiated. Hearts were perfused at a constant pressure of 35-40 mm Hg for 150 minutes at which time the hearts were freeze-clamped by tongs cooled in liquid nitrogen.

Extraction of the Tissues. The frozen tissues were pulverized with a mortar and pestle cooled to dry ice temperature in a cold room at 4°C. The pooled chick brain or rat heart samples [2 gm] were weighed onto frozen 10% TCA [1 volume] in plastic test tubes over dry ice and brought to 4°C by the addition of another volume of ice cold 10% TCA. The samples were homogenized at 4°C with a Potter-Elvehjem homogenizer with Teflon pestle. The homogenate was centrifuged at 10,000 x g, 15 minutes at 4°C in a SS-34 rotor of a Sorvall RC 2-B centrifuge. The pellet was rehomogenized with an additional 2 volumes of ice cold 10% TCA and centrifuged as before. The supernatant fractions were combined in a 50 ml heavy wall Pyrex centrifuge tube and placed [covered with a marble] in a boiling water bath for 20 minutes. Control experiments with galactose 1-phosphate, galactose 6-phosphate and with [U-^{14}C]-galactose 1-phosphate demonstrated that galactose phosphorylated in the 6 position was fully stable [see Appendix]. The hydrolysates were cooled and the TCA was removed by 5 repetitive extractions with 2 volumes of diethyl ether. Residual ether was removed by nitrogen aeration. The aqueous solution was adjusted to

pH 7.0 with 2 N KOH and reduced to approximately 0.5 ml by a rotary evaporator. The extract was applied to the origin of Whatman 3 MM paper sheets [15 x 15 inches] and chromatographed ascendingly in jars containing ethanol:1 M ammonium acetate buffer, pH 7.5 [7:3, v/v] for 18-24 hours. In this solvent system, neutral sugars can be easily separated from the slower moving hexose monophosphates. Hexose diphosphates remain very near the origin.

The hexose mono- and diphosphate zones were detected by procedures described previously (27,28) and by cochromatography with [U-^{14}C]-glucose 6-phosphate and detection with a Packard Model 7201 radiochromatogram scanner. The hexose phosphate band was cut from the paper chromatogram and the paper was extracted three times with deionized water at 90°C. The combined aqueous extract was treated at room temperature with 1-2 gm of Dowex-50 [H^+], 50-100 mesh, to remove extraneous cations. The resulting solution was adjusted to a suitable volume and a pH of 7.0 with 2 N KOH prior to analytic studies. The diphosphate region of the paper chromatogram was cut out and an extract of the paper prepared as described above. We have considered the possibility that galactose 1,6-diphosphate could produce erroneously high galactose 6-phosphate levels after hydrolysis of the 1-phosphate in the presence of TCA. Accordingly, identical extractions were prepared except the boiling water bath treatment was omitted. The diphosphate region of the paper chromatogram was cut out and an extract of the paper prepared as in

the case of the monophosphate band. Essentially no galactose was liberated after the addition of alkaline phosphatase in a reaction mixture containing NAD^+ and β-galactose dehydrogenase ruling out this potential source of the galactose 6-phosphate found in the tissues.

Metabolite Determinations. Glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate eluted from the hexose mono- and diphosphate region of the paper chromatogram were determined by slight modifications of the procedures of Lowry et al. (29). Galactose 6-phosphate was analyzed by the successive addition of β-galactose dehydrogenase and E. coli alkaline phosphatase (E.C. 3.1.3.1). The reaction mixture consisted of 100 mM Tris-HCl, pH 8.1; 0.5 mM NAD^+; 10 µl of β-galactose dehydrogenase [25 units/ml]; 5 µl E. coli alkaline phosphatase [400 units/ml], and extract in a final volume of 0.5 ml. The amount of NADH formed in the reaction representing stoichiometric quantities of galactose was determined in a Model 2400-S Gilford spectrophotometer at 340 nm and 25°C. β-Galactose dehydrogenase from Pseudomonas fluorescens was inactive toward the following sugars: D-fructose, D-mannose, D-lyxose, D-xylose, D-ribose, N-acetylgalactosamine, and < 1.0% activity with D-galactosamine. L-Arabinose reacted as rapidly as D-galactose. The reduction of NAD^+ commenced upon the addition of alkaline phosphatase releasing galactose and was virtually complete after 60 minutes. When [U-^{14}C]-glucose 6-phosphate was added to the original tissue

homogenates, a recovery of 30-31% was achieved over the total process permitting appropriate corrections to be made for hexose phosphates [see Appendix]. The greatest losses [ca. 1/3] occurred during the extraction of the TCA. Galactose, galactitol, and glucose were determined by gas-liquid chromatography (30).

Combined Gas-Liquid Chromatography-Mass Spectrometry. Aliquots of the purified tissue extracts were dried in 50 ml centrifuge tubes and trimethylsilylated with a mixture of pyridine:bis-trimethylsilylacetamide:trimethylchlorosilane [2:5:0.5, v/v/v] by warming the reaction to 90°C for 30-60 minutes.

Mass spectra were recorded at 70 eV with a LKB 9000 mass spectrometer, and the relative abundance of fragments was displayed as bar graphs by means of an on-line data acquisition and processing program (31). The source temperature was 290°C, accelerating voltage 3.5 kV, and the ionizing current 60 uA. Sample introduction was via the GC inlet using a 4 foot, 3 mm I. D. glass coil packed with 1% OV-1 on Gas-Chrom Z [100-120 mesh]. The column temperature was 200°C.

Hexose 6-Phosphate Dehydrogenase

Enzyme Preparations. Liver [right lobe], heart and brain samples were removed from animals sacrificed by decapitation. One gram of rat liver, heart, brain, or chick brain was homogenized in 5 volumes of ice cold, 0.25 M sucrose in a Potter-Elvehjem type homogenizer. The homogenates were centrifuged at 1,000 x g at 4°C for 30 minutes in a Sorvall RC-2B

centrifuge with an SS-34 rotor. The pellets were discarded and their supernates were recentrifuged at 10,000 x g for 30 minutes. The pellets were rinsed twice with a few ml of ice cold 0.25 M sucrose, then resuspended with a Potter-Elvehjem homogenizer in 2.0 ml of 0.25 M sucrose and 0.5 ml, 10% [v/v] Triton X-100. The fractions represented crude mitochondrial fractions [about 3 mg/ml liver protein, 2 mg/ml heart protein, or 4 mg/ml brain protein]. The 10,000 x g supernates were recentrifuged at 100,000 x g at 4°C for 2 hours in a Beckman Model L ultracentrifuge with a 40 K rotor. The pellets from liver and heart preparations were rinsed and resuspended as were the mitochondrial fractions. The smaller pellets from brain preparations were resuspended in 1.0 ml 0.25 M sucrose and 0.25 ml, 10% Triton X-100. These fractions represented crude microsomal fractions [about 7 mg/ml liver protein, 2 mg/ml heart protein, or 3 mg/ml brain protein]. The 100,000 x g supernates were designated the soluble fractions [about 15 mg/ml liver protein, 5 mg/ml heart protein, or 4 mg/ml brain protein].

Assay Procedure. The reaction was initiated by the addition of glucose 6-phosphate or galactose 6-phosphate and was performed at 25°C. The standard incubation mixture contained Tris-HCl, pH 10.0 [100 mM], $NADP^+$ [1.5 mM], or NAD^+ [1.0 mM], glucose 6-phosphate [2.0 mM], or galactose 6-phosphate [2.0 mM], 50 µl of a subcellular enzyme preparation, and water to a final volume of 0.5 ml. The reaction rate was measured by the increase in absorbance at 340 nm with

time. Kinetic analyses of the $NADP^+$-dependent oxidation of galactose 6-phosphate by rat liver microsomal and rat brain soluble fractions were performed over a substrate range of 0.1 mM to 5.0 mM galactose 6-phosphate.

In Vitro Synthesis of 6-Phospho-D-Galactonate

Rat liver microsomal and rat brain soluble fractions were prepared as described for hexose 6-phosphate dehydrogenase. Two ml of brain soluble fraction or 1.5 ml of liver microsomal fraction were added to an incubation mixture in a 50 ml Pyrex centrifuge tube containing Tris-HCl, pH 10.0 [100 mM], galactose 6-phosphate [5.0 mM] and $NADP^+$ [3.0 mM] in a final volume of 5.0 ml. Samples of 1.5 ml of each reaction mixture were removed and boiled for 5 minutes in a boiling water bath to serve as zero time controls. Aliquots [1 ml] were treated with 50 µl of alkaline phosphatase and incubated for 4 hours at 25°C. Samples [50 µl] of the zero time controls, with or without alkaline phosphatase treatment were evaporated at 40°C. The residues were trimethylsilylated with 100 µl of a mixture of pyridine:bis-trimethylsilylacetamide:trimethylchlorosilane [2:5:0.5, v/v/v]. The remaining 3.5 ml of each incubation mixture was incubated at 37°C for 90 minutes. The reactions were stopped by boiling for 5 minues in a boiling water bath, and then 3.0 ml of each sample were treated with alkaline phosphatase as described above. Fifty microliters of each fraction, with or without alkaline phosphatase treatment, were dried and

trimethylsilylated as described above. Gas liquid chromatography (30) was performed at 190°C on a 3 mm x 1.8 M column packed with 3% OV-1 on Chromosorb W, 100-200 mesh. Galactose, galactono-γ-lactone, glucono-δ-lactone, sodium galactonate, and sodium gluconate standards were analyzed for peak retention time identifications. Lactones prepared in 0.1 M sodium hydroxide yielded the sodium salts of galactonic and gluconic acids.

Synthesis of 6-Phospho-D-Galactonic Acid

6-Phosphogalactonic acid was prepared by the bromine oxidation of galactose 6-phosphate (32). Barium D-galactose 6-phosphate [2 gm] was dissolved in 14.0 ml of distilled water in a 50 ml Pyrex centrifuge tube. Insoluble material was sedimented at 1,000 rpm. Barium carbonate [2.4 gm] and bromine [0.3 ml] were added to the supernate in a 50 ml ground glass stoppered Erlenmeyer flask and shaken at room temperature for 2 hours. Bromine was removed by nitrogen aeration, and the barium carbonate was filtered from the solution and washed with water. The filtrate and washings were adjusted to pH 3.5 with glacial acetic acid and the barium salt of the oxidation product was precipitated by adding 100 ml of absolute ethanol at ice temperature. The precipitate was filtered, washed with 200 ml of 80% ethanol, and dried overnight *in vacuo* over calcium chloride. The dried precipitate was redissolved in 100 ml of distilled water containing a few drops of 1% phenolphthalein in ethanol. The solution

was aerated with nitrogen and saturated barium hydroxide was added until a permanent pink end point was reached. Fifty ml of absolute ethanol was added and after 1 hour at room temperature, the precipitate was filtered with suction, washed with absolute ethanol, and dried in vacuo over calcium chloride. Approximately 1 gm of barium 6-phospho-D-galactonic acid was obtained. Prior to use, the barium was exchanged with potassium from Dowex-50. Analysis of the synthetic 6-phosphogalactonate showed a 1:1 stoichiometry between inorganic phosphate and the sum of galactonic acid and galactono-γ-lactone after treatment with alkaline phosphatase. Spectrophotometric analysis of the compound with coupled systems of β-galactose dehydrogenase, alkaline phosphatase, and NAD^+ or glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and $NADP^+$ showed no detectable galactose, galactose phosphate, glucose 6-phosphate, or 6-phosphogluconate. Gas liquid chromatography (30) of the trimethylsilylated derivative showed a single peak with a slightly shorter retention time than 6-phosphogluconic acid [see Appendix for description of characterization].

Oxidation of 6-Phospho-D-Galactonic Acid

Rat brain crude mitochondrial, microsomal and soluble fractions were prepared as described for hexose 6-phosphate dehydrogenase except that 0.32 M sucrose containing 1 mM β-mercaptoethanol was used as the homogenizing media. The reaction was initiated at 25°C by the addition of

6-phosphogalactonate [2.0 mM] to a cuvette containing HEPES buffer [40 mM, pH 7.5], $MgCl_2$ [6.7 mM], thioglycerol [10.2 mM], $NADP^+$ [1.5 mM], or NAD^+ [2.0 mM], 50 µl of a subcellular enzyme preparation in a final volume of 0.5 ml. The course of the reaction was monitored by the change in absorbance at 340 nm with time.

Galactose Phosphate Alkaline Phosphatase

One gram of rat heart or brain was homogenized in 5 volumes of ice cold 0.32 M sucrose with 1 mM β-mercaptoethanol in a Potter-Elvehjem homogenizer. The homogenates were contrifuged at 1,000 x g for 30 minutes at 4°C in a Sorvall RC-2B with an SS-34 rotor. The supernates were recentrifuged at 1,000 Kg for 30 minutes and the final supernates were made 1% [v/v] with Triton X-100. These crude fractions were used as the source of galactose phosphate phosphatase. The reaction was initiated at 25°C by the addition of enzyme preparation to a cuvette containing Tris-HCl, pH 9.0 [100 mM], NAD^+ [1 mM], β-galactose dehydrogenase (5 µl, 5 mg/ml], galactose 6-phosphate or galactose 1-phosphate in a final volume of 0.5 ml. The course of the reaction was followed by the increase in absorbance at 340 nm with time. Assays were performed over a substrate range of 0.25 mM to 5.0 mM galactose phosphate.

6-Phospho-Galactonate Alkaline Phosphatase

Rat brain crude enzyme fractions were prepared as described for galactose-phosphate phosphatase. The reactions were initiated by the addition of 60 µl of the enzyme preparation to small Pyrex culture tubes containing Tris-HCl, pH 9.0 [100 mM], potassium 6-phosphogalactonate [0.1 mM to 5.0 mM] in a final volume of 0.2 ml. The tubes were incubated at 37°C for 150 minutes in a Dubnoff metabolic incubator and the reactions were stopped by the addition of 0.1 ml water and 1.2 ml of acid molybdate reagent for the quantitation of inorganic phosphate (33).

Glycolytic and Hexose Monophosphate Shunt Enzyme Assays

Phosphoglucose Isomerase. The assay procedure of Lowry and Passoneau (34) was used with minor modifications. One gram of rat brain was homogenized in 10 volumes of ice cold 0.32 M sucrose with 1 mM β-mercaptoethanol in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,000 x g for 30 minutes at 4°C in a Sorvall centrifuge with an SS-34 rotor. The supernate was centrifuged at 10,000 x g for 30 minutes and its supernate recentrifuged at 10,000 x g for 30 minutes. The final supernate was diluted 1:10 with homogenizing media for assaying. The reaction was initiated at 25°C by adding glucose 6-phosphate [0.02 mM to 1.0 mM] to a cuvette containing imidazole buffer, pH 7.2 [20 mM], potassium acetate [150 mM], $MgCl_2$ [5 mM], mono-basic potassium phosphate [5 mM], bovine serum albumin

[0.02%, w/v], NADH [0.2 mM], ATP [2.5 mM], AMP [0.05 mM], fructose 6-phosphate kinase [1 µl, 10 mg/ml], aldolase [1 µl, 10 mg/ml], α-glycerol phosphate dehydrogenase [1 µl, 1 mg/ml], triose phosphate isomerase [1 µl, 10 mg/ml], 25 µl of brain enzyme preparation, and water to a final volume of 0.5 ml. The reaction course was followed by the decreasing absorbance at 340 nm with time. The effects of galactose 6-phosphate at 0.2 mM or 1.0 mM and 6-phosphogalactonate at 0.4 mM were tested at all glucose 6-phosphate concentrations.

Phosphoglucomutase. The enzyme preparation and assay were the same as those described for phosphoglucose isomerase except that the enzyme preparation was not diluted 1:10 but assayed directly, phosphoglucose isomerase [1 µl, 11 mg/ml] was added to the reaction mixture, and the reaction was initiated with glucose 1-phosphate over a concentration range of 0.02 mM to 1.0 mM. The effects of galactose 6-phosphate and 6-phosphogalactonate were also tested as described.

Glucose 6-Phosphate Dehydrogenase. The enzyme preparation was the same as that described for phosphoglucomutase. The assay was initiated at 25°C by the addition of glucose 6-phosphate [0.02 mM to 1.0 mM] to a cuvette containing imidazole buffer, pH 7.2 [20 mM], potassium acetate [150 mM], $MgCl_2$ [5 mM], mono-basic potassium phosphate [5 mM], bovine serum albumin [0.02%, w/v], $NADP^+$ [1.5 mM], 6-phosphogluconate dehydrogenase [1 µl, 2.5 mg/ml], 25 µl of enzyme preparation in a final volume of 0.5 ml. The reaction rate was monitored by the increasing absorbance at 340 nm with time.

Effects of galactose 6-phosphate and 6-phosphogalactonate were tested as described under phosphoglucose isomerase.

6-Phosphogluconate Dehydrogenase. The enzyme preparation and assay were the same as those described for glucose 6-phosphate dehydrogenase except that 6-phosphogluconate dehydrogenase was not added and the reaction was initiated with 6-phosphogluconate [0.02 mM to 1.0 Mm]. The effect of 6-phosphogalactonate at 0.4, 0.8, 1.2 and 1.6 mM was tested at all 6-phosphogluconate concentrations.

Soluble Hexokinase. The enzyme preparation was the same as that described for phosphoglucomutase except that following the first 10,000 x g centrifugation, the supernates were recentrifuged at 100,000 x g at 4°C for 60 minutes in a Beckman Model L ultracentrifuge with a 40K head. The reaction was initiated at 25°C by the addition of glucose [0.01 mM- 0.50 mM] to a cuvette containing HEPES buffer, pH 7.5 [40 mM], $MgCl_2$ [6.7 mM], thioglycerol [10.2 mM], ATP [5.0 mM], NADH [0,2 mM], AMP [0.05 mM], phosphoglucose isomerase [1 µl, 11 mg/ml], fructose 6-phosphate kinase [1 µl, 10 mg/ml], aldolase [1 µl, 10 mg/ml], triose phosphate isomerase [1 µl, 10 mg/ml], α-glycerol dehydrogenase [1 µl, 10 mg/ml], 25 µl of enzyme preparation in a final volume of 0.5 mM. The course of the reaction was followed by the decrease in absorbance at 340 nm with time. The effect of galactose 6-phosphate at 0.2 mM or 1.0 mM was tested at all glucose concentrations. Protein was determined by the method of Lowry (35) using bovine serum albumin as standard.

Results

Tissue Galactose 6-Phosphate

Chick Brain Metabolites. As previously observed (3, 36, 37), several glycolytic intermediates were markedly reduced in the brains of galactose-intoxicated chicks (Table 3). Glucose 6-phosphate was 25% of control levels [35.5 ± 10.3 versus 140.0 ± 19.3 nmoles/gm] and fructose 6-phosphate and fructose 1,6-diphosphate were likewise severely decreased. As expected, galactose 1-phosphate was found in significant amounts, 585.8 nmoles/gm, as a result of consuming a galactose-containing diet, whereas galactose 6-phosphate was estimated to be 17.1 ± 3.0 nmoles/gm. Galactose 1-phosphate was observed in the brains of chicks fed a control diet at levels closely similar to the corresponding glucose ester (Table 3) [65.2 ± 4.7 versus 63.4 ± 2.1 nmoles/gm].

Galactose Perfused Rat Hearts. Hearts perfused with 30 mM galactose for 150 minutes showed marked depression in rate of contraction. The key glycolytic metabolites were severely depressed (Table 4) and galactose metabolites were elevated. Glucose 6-phosphate was reduced to 9.5 ± 3.8 nmoles/gm while fructose 6-phosphate and fructose 1,6-diphosphate were present in less than detectable amounts. Galactose 6-phosphate levels were greater than glucose 6-phosphate [20.4 ± 1.5 versus 9.5 ± 3.8 nmoles/gm] and glucose was undetectable. Galactose 1-phosphate and galactitol were detected at levels of 389.7 nmoles/gm and 1.19 ± 0.7 μmoles/gm, respectively.

Galactose 1-phosphate in glucose perfused hearts was present in levels similar to glucose 6-phosphate (Table 4) [94.6 ± 17.2 versus 83.6 ± 27.4 nmoles/gm].

Gas Chromatographic Analysis. Fully trimethylsilylated D-galactose 6-phosphate is a mixture of 2 furanose and 2 pyranose forms (Figure 14, peaks 1-4) (38) whereas glucose 6-phosphate exists as α and β-pyranose forms (Figure 14, peaks 5 and 6). Gas chromatography of the derivatives of the extracts from chick brain revealed peaks with the retention times of trimethylsilylated α and β-galactofuranoside 6-phosphate (Figure 14, peaks 1 and 2), and trimethylsilylated α and β-galactopyranoside 6-phosphate (Figure 14, peaks 3 and 4).

Mass Spectra. The mass spectra of the TMS derivatives of authentic β-D-galactopyranoside 6-phosphate (Figure 14, peak 4) is presented for comparison above the spectrum for peak 4 from the chick brain extract (Figure 15). Its fragmentation pattern is virtually identical with that published by Harvey et al. (38) for β-D-galactopyranoside 6-phosphate with M-15 at 677 and characteristically abundant ions for aldohexose 6-phosphates of m/e 387 and 357. All the characteristic ions are evident and add strong support to the presence of galactose 6-phosphate in brains of chicks fed galactose.

Hexose 6-Phosphate Dehydrogenase

The subcellular distribution of $NADP^+/NAD^+$-dependent glucose 6-phosphate/galactose 6-phosphate dehydrogenase

activity [hexose 6-phosphate dehydrogenase] is listed in Table 5. $NADP^+$-dependent glucose 6-phosphate dehydrogenase activity [G6PD] was found in all fractions except rat heart microsomes. Since the assay does not distinguish between glucose 6-phosphate or hexose 6-phosphate dehydrogenases when glucose 6-phosphate [G6P] and $NADP^+$ are substrate and cofactor, the $NADP^+$-dependent oxidation of galactose 6-phosphate [Gal-6-P] or the NAD^+-dependent oxidation of G6P and Gal-6-P are taken as a measure of hexose 6-phosphate dehydrogenase [H6PD] activity. From Table 5, H6PD activity is therefore seen to be primarily associated with the particulate fractions of rat liver. Since H6PD is a microsomal enzyme (39), the soluble $NADP^+$-dependent Gal-6-P dehydrogenase activity of rat brain is not attributed to H6PD. The capacity of liver microsomal or mitochondrial fractions to oxidize Gal-6-P with $NADP^+$ as cofactor was about 50% of that using G6P as substrate. Also, with either G6P or Gal-6-P as substrate, $NADP^+$ was a better oxidant than NAD^+. NAD^+-dependent G6P or Gal-6-P dehydrogenase activity was undetectable in rat brain, heart, and chick brain. $NADP^+$-dependent Gal-6-P dehydrogenase was not found in rat heart or chick brain. Rat brain soluble $NADP^+$-dependent Gal-6-P dehydrogenase activity was further characterized by comparing it kinetically with rat liver microsomal H6PD (Figure 16). While the liver enzyme had a Km for Gal-6-P of 0.5 mM, the brain enzyme had a much higher Km of about 10 mM.

In Vitro Synthesis of 6-Phosphogalactonic Acid by Rat Liver Microsomal and Rat Brain Soluble Fractions

In order to identify the products of the $NADP^+$-dependent Gal-6-P dehydrogenase activities of rat liver and brain, liver microsomal and brain soluble fraction were incubated for 90 minutes with $NADP^+$ and Gal-6-P as described under Materials and Methods. Since 6-phosphogalactonate [6-P-Gala] could not be separated from the α-pyranose form of Gal-6-P by the gas liquid chromatographic system employed, [see Appendix] samples of the reaction mixtures were treated with alkaline phosphatase. Under these conditions, the galactose and galactonate liberated from the substrate and product, respectively, could be easily separated [see Appendix]. Gas liquid chromatography of the products of the alkaline phosphatase treated samples prior to the 90 minute incubation period are shown in the upper tracings A of Figure 17. Peaks 1, 2, and 3 were identified from standards as α-D-galactofuranose, α, and β-D-galactopyranose, respectively. The 4th peak was an unknown whose presence was independent of the incubation period or the alkaline phosphatase treatment. Following the incubation period, and after alkaline phosphatase treatment (Figure 17, tracings B), a new peak (#5) with the same retention time as trimethylsilylated sodium D-galactonate appeared. Gas liquid chromatography of incubated and non-incubated samples which were not treated with alkaline phosphatase are shown in tracings C and D. The amount of 6-P-Gala synthesized by the liver

preparation during the incubation period was approximately twice that produced by the brain.

Oxidation of 6-Phosphogalactonic Acid

Rat brain mitochondrial, microsomal, and soluble fractions were assayed for 6-P-Gala dehydrogenase activity as described under Materials and Methods. No measurable $NADP^+$- or NAD^+-dependent oxidation of 6-P-Gala could be found in the subcellular fractions.

Galactose-Phosphate Alkaline Phosphatase

Kozak et al. (2) have described an alkaline phosphatase activity in chick brain which will hydrolyze galactose 1-phosphate. To determine whether Gal-6-P could be hydrolyzed by an alkaline phosphatase activity in rat brain and heart, crude preparations from these tissues were assayed for both Gal-6-P and galactose 1-phosphate [Gal-1-P] phosphatases. Double reciprocal plots of these activities in brain are shown in Figure 18. The maximal rates of hydrolysis for both galactose phosphates were similar [about 20 nmoles galactose released/min/gm tissue]. The Km for Gal-1-P and Gal-6-P hydrolysis were 1 mM and 2 mM, respectively. Neither Gal-1-P nor Gal-6-P phosphatase activities could be detected in rat heart enzyme preparations.

6-Phosphogalactonate Alkaline Phosphatase

Since 6-P-Gala was not oxidized by rat brain subcellular fractions, the possibility of its dephosphorylation to galactonate was investigated. Crude preparations from rat

brain were assayed over a substrate range of 0.1 to 5.0 mM 6-P-Gala for phosphatase activity (Figure 19). A maximal velocity [about 40 nmoles Pi released/min/gm tissue] was reached by 2 mM 6-P-Gala beyond which the substrate appeared inhibitory.

Effect of Galactose 6-Phosphate and 6-Phosphogalactonate on Glycolytic and Hexose Monophosphate Shunt Enzyme

Galactose 6-phosphate and 6-phosphogalactonate were tested as potential inhibitors of rat brain soluble hexokinase, phosphoglucomutase, phosphoglucose isomerase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase as described under Materials and Methods. Although Gal-6-P did not affect any of the enzymes tested (Figures 20, 21 and 22), 6-P-Gala competitively inhibited 6-phosphogluconate dehydrogenase (Figure 23). A Dixon analysis of this inhibition yielded a Ki of 0.5 mM for 6-phosphogalactonate (Figure 24).

Discussion

Investigations with galactose fed rats (40) and galactosemic blood cells (41) have failed to detect galactose 6-phosphate. However, the tissue galactose concentrations achieved in these studies were less than 6 mM. Studies by Inouye (42) with galactosemic erythrocytes incubated with higher galactose levels [10-30 mM] showed that small amounts of galactose 6-phosphate were produced. We have also found

evidence for the presence of low levels of galactose 6-phosphate in the brains of chicks fed galactose-enriched diets and rat hearts perfused with high concentrations of galactose. While tissue galactose 6-phosphate levels were comparable to certain glycolytic metabolites in the galactose treated animals, galactose 6-phosphate only comprised about 5% of the total galactose phosphates.

Since others (11, 12) have suggested that the residual oxidation of galactose observed in classic galactosemia may arise from the formation and oxidation of galactose 6-phosphate, studies on its metabolism in rat heart, brain, liver and chick brain were undertaken. Rat liver hexose 6-phosphate dehydrogenase [H6PD] has been described by Beutler et al. to be membrane associated (39). In agreement, we find the $NADP^+$-dependent oxidation of Gal-6-P as well as the NAD^+-dependent oxidation of G6P and Gal-6-P, primarily in the mitochondrial and microsomal fractions (Table 5). H6PD activity was not found in any subcellular fraction of rat brain, heart, or chick brain although an $NADP^+$-dependent oxidation of Gal-6-P was found in the soluble fraction of rat brain. The absence of a similar activity in rat brain mitochondrial and microsomal fractions suggest that the oxidation of Gal-6-P was not due to H6PD activity. Since glucose 6-phosphate dehydrogenase [G6PD] can convert galactose 6-phosphate to 6-P-Gala in the presence of $NADP^+$ (11, 43), a more likely explanation is that the oxidation of Gal-6-P is due to the high soluble G6PD activity of rat brain.

In good agreement with the literature (39), a Km value of 0.5 mM for Gal-6-P was calculated for liver microsomal H6PD (Figure 16). However, a much higher Km of 10 mM was calculated for brain soluble Gal-6-PD which further illustrates the difference between the two activities. Kirkman has reported a Km of 2.3 mM for galactose 6-phosphate for erythrocyte glucose 6-phosphate dehydrogenase (43). Despite differences in subcellular localization and kinetics of liver H6PD and brain Gal-6-PD, the reaction product of both activities was 6-P-Gala (Figure 17).

Srivastava and Beutler have investigated the conversion of ^{14}C labeled 6-P-Gala to $^{14}CO_2$ by rat liver and have been unable to demonstrate either a pyridine or flavin nucleotide-dependent oxidation (12). Similarly, we found neither $NADP^+$ nor NAD^+-dependent oxidation of 6-P-Gala in the subcellular fractions of rat brain. This excludes the possibility that Gal-6-P could be oxidized to carbon dioxide and pentose phosphates through a 6-P-Gala intermediate in brain. If 6-P-Gala were to form in brain, it would likely be dephosphorylated by alkaline phosphatase activity (Figure 19).

Galactose 1-phosphate hydrolysis to galactose and inorganic phosphate has been implicated in a futile cycle which may contribute to a reduction in cellular ATP levels (23, 24). In this report, we have described an alkaline phosphatase activity in rat brain which dephosphorylates both Gal-1-P and Gal-6-P (figure 18). Since tissue concentrations of Gal-6-P are much lower than Gal-1-P, the hydrolysis of Gal-6-P would

seem to be of secondary importance to the hydrolysis of Gal-1-P in the depletion of ATP reserves by the futile cycle.

Galactose 6-phosphate and 6-P-Gala were tested as potential inhibitors of several glycolytic and hexose monophosphate shunt enzymes. The only enzyme affected was 6-phosphogluconate dehydrogenase (Figure 23). Since the Ki calculated for 6-P-Gala was high [0.5 mM], it is unlikely that 6-P-Gala could influence 6-phosphogluconate dehydrogenase *in vivo*.

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Table 3. Comparison of Selected Metabolites in the Brains of Chicks Fed Either a Control or Galactose-Containing Diet.

Metabolite	Chick Brain	
	Control	Galactose-Fed
	nmoles/gm tissue	
Glucose 1-phosphate	63.4 ± 2.1	69.7
Glucose 6-phosphate	140.0 ± 19.3	35.5 ± 10.3
Fructose 6-phosphate	58.3 ± 28.4	<3.0[1]
Fructose 1,6-diphosphate	32.2 ± 12.3	4.5 ± 2.0
Galactose 1-phosphate	65.2 ± 4.7	585.8[2]
Galactose 6-phosphate	<3.0[1]	17.1 ± 3.0
	µmoles/gm tissue	
Galactose	[3]	6.0 ± 0.4
Galactitol	[3]	15.1 ± 0.9
Glucose	2.5 ± 0.3	[3]

Dietary treatments are as described under Materials and Methods. The values represent the mean ± S. D. for 3 pools of 10 brains for the control group and 3 pools of 5 brains for the galactose-fed group.

[1]Lower limit of sensitivity.

[2]Expressed as the difference between total galactose phosphate and galactose 6-phosphate.

[3]Undetectable by gas-liquid chromatography.

Table 4. Comparison of Selected Metabolites in Rat Hearts Perfused with Media Containing Either Glucose or Galactose.

Metabolite	Perfused Rat Heart	
	Glucose	Galactose
	nmoles/gm tissue	
Glucose 6-phosphate	83.6 ± 27.4	9.5 ± 3.8
Fructose 6-phosphate	12.3 ± 3.4	<3.0[1]
Fructose 1,6-diphosphate	<3.0[1]	<3.0[1]
Galactose 1-phosphate[2]	94.6 ± 17.2	389.7
Galactose 6-phosphate	<3.0[1]	20.4 ± 1.5
	µmoles/gm tissue	
Galactose	[3]	29.8 ± 0.2
Galactitol	[3]	1.2 ± 0.1
Glucose	1.3 ± 0.04	[3]

The condition of heart perfusion is described under Materials and Methods. The values represent the mean ± S. D. for 3 pools of 4 hearts.

[1]Lower limit of sensitivity.

[2]Expressed as the difference between total galactose phosphate and galactose 6-phosphate.

[3]Undetectable by gas-liquid chromatography.

Table 5. Subcellular Distribution of $NADP^+$ and NAD^+-Dependent Glucose 6-Phosphate and Galactose 6-Phosphate Dehydrogenase Activities in Rat Liver, Heart, Brain, and Chick Brain.

Tissue and Subcellular Fraction	Glucose 6-Phosphate		Galactose 6-Phosphate	
	$NADP^+$	NAD^+	$NADP^+$	NAD^+
Rat Liver				
Mitochondrial	5.94	0.64	2.99	0
Microsomal	4.95	0.89	2.87	0.76
Soluble	9.10	0	0.65	0
Rat Brain				
Mitochondrial	11.44	0	0	0
Microsomal	8.56	0	0	0
Soluble	36.60	0	0.89	0
Rat Heart				
Mitochondrial	0.91	0	0	0
Microsomal	0	0	0	0
Soluble	4.45	0	0	0
Chick Brain				
Mitochondrial	2.24	0	0	0
Microsomal	2.44	0	0	0
Soluble	3.14	0	0	0

Assay methods are described in Materials and Methods under hexose 6-phosphate dehydrogenase. Activities are expressed as nmoles glucose 6-phosphate or galactose 6-phosphate oxidized by $NADP^+$ or NAD^+/min/mg protein. The values are the average of 2 separate experiments.

Figure 14. Gas chromatographic separation of galactose 6-phosphate and glucose 6-phosphate standards and chick brain extracts.

Trimethylsilyl derivatives of standard galactose 6-phosphate (peaks 1-4) and glucose 6-phosphate (peaks 5 and 6)[lower trace] and chick brain extracts [upper trace] on 1% OV-1 [4 ft.] at 200°C. The mass spectra presented in Figure 15 were taken from the corresponding standard β-D-galactopyranosyl 6-phosphate and chick brain extract (peaks 4).

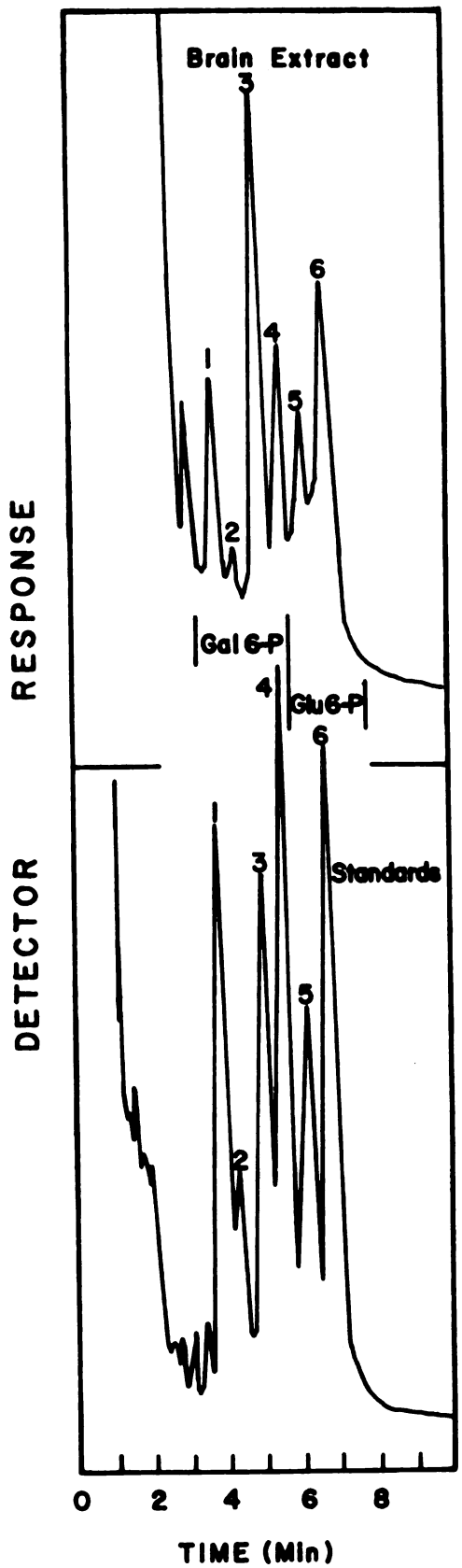

Brain Extract
3
6
4
1
5
2
Gal 6-P
4
Glu 6-P
6
DETECTOR RESPONSE
1
3
Standards
5
2
0
2
4
6
8
TIME (Min)

Figure 15. **Comparative mass spectra of chick brain and standard β-D-galactopyranosyl 6-phosphate.**

Mass spectra of the trimethlsilyl derivatives of standard β-D-galactopyranosyl 6-phosphate [upper plot] and the corresponding derivative from chick brain extract [lower plot] were recorded at 70 eV. The derivatives are represented in Figure 14 as peak 4.

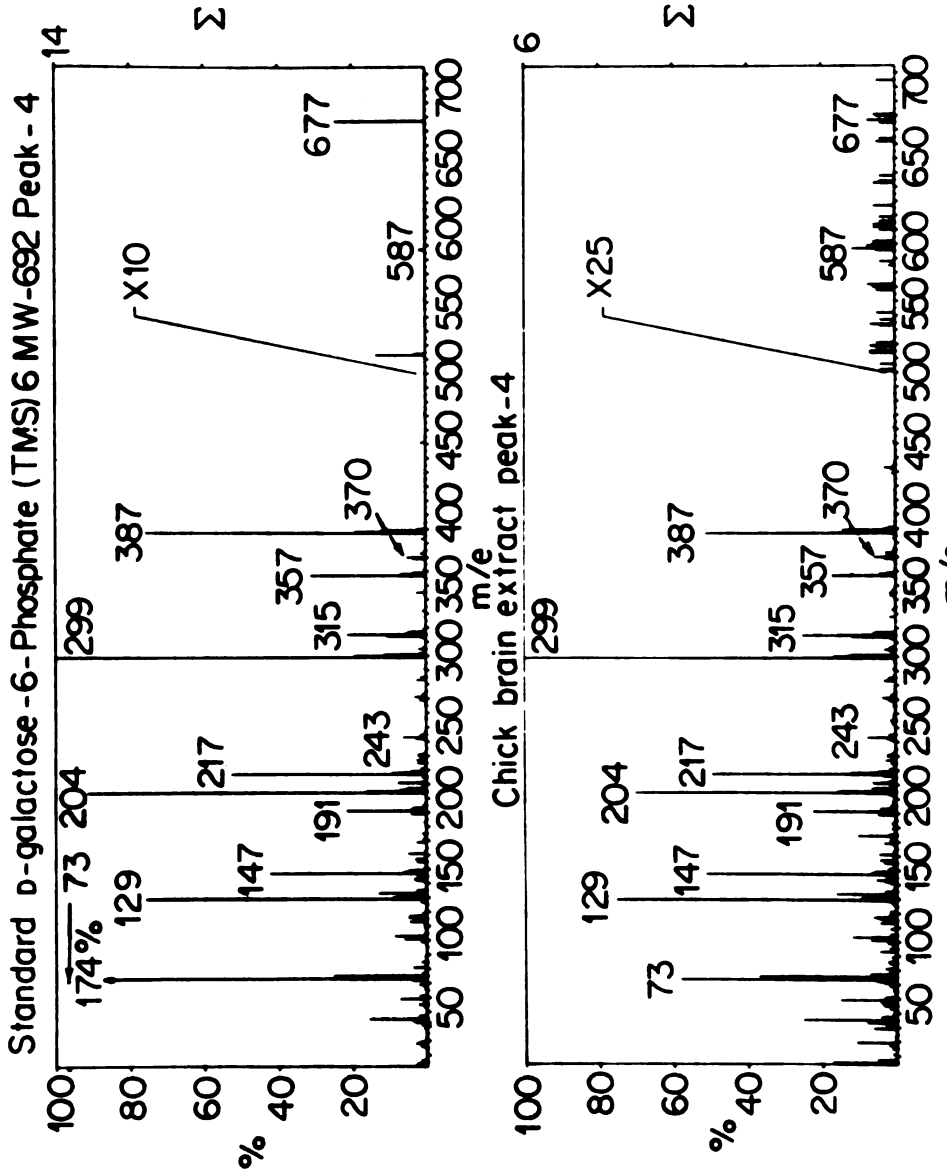

Standard D-galactose-6-Phosphate (TMS)6 MW-692 Peak-4
Chick brain extract peak-4
Σ
X10
X25
m/e
%
73
129
147
174
191
204
217
243
299
315
357
370
387
587
677

Figure 16. A kinetic comparison of rat liver microsomal and rat brain soluble $NADP^+$-dependent galactose 6-phosphate dehydrogenase activities.

A substrate-velocity plot for liver microsomal O——O and brain ●——● soluble galactose 6-phosphate dehydrogenase activity with varying galactose 6-phosphate concentrations. Velocity is given in nmoles Gal-6-P oxidized/min/mg protein. The assays were performed as described in Materials and Methods.

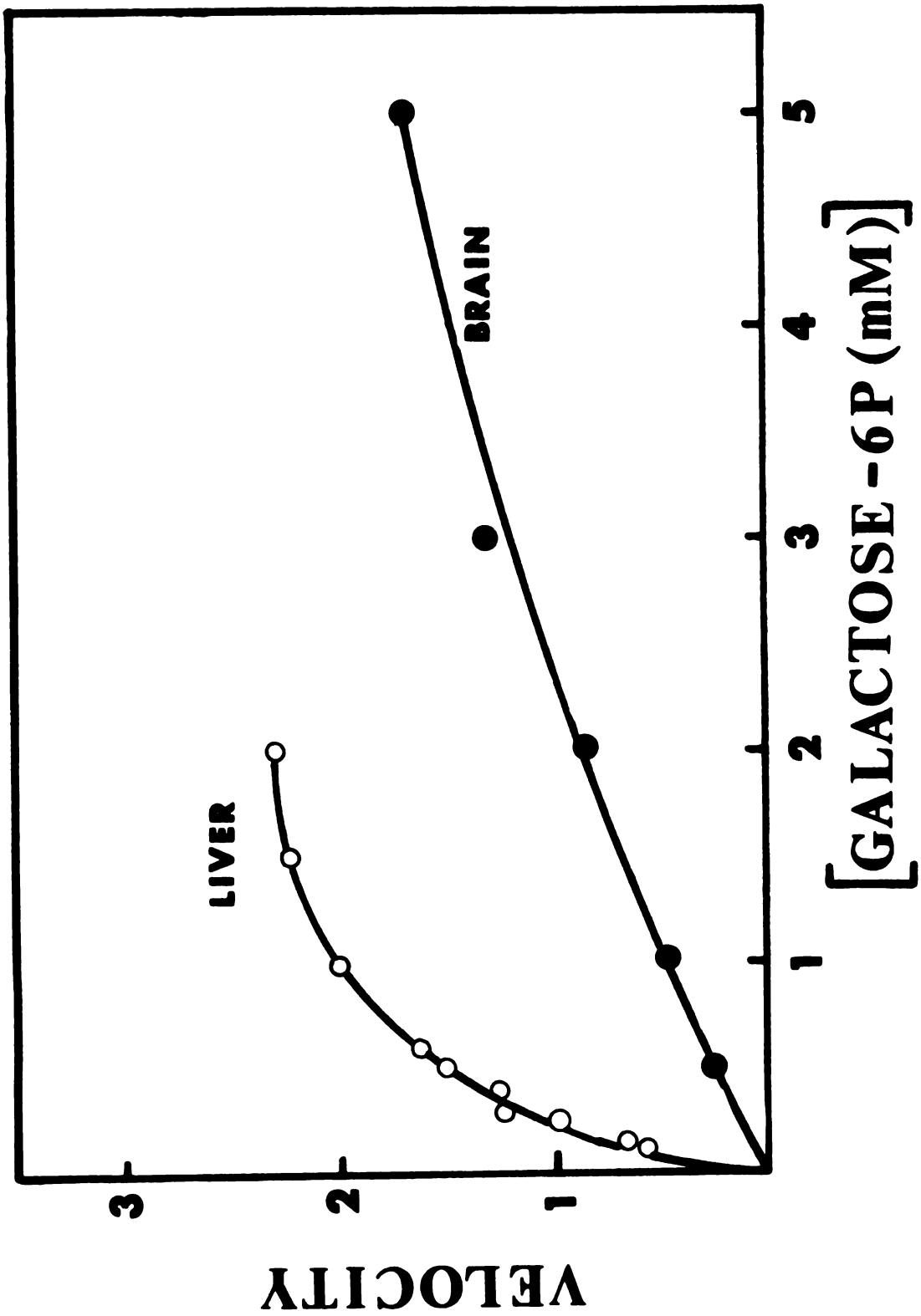

VELOCITY
3
2
1
LIVER
BRAIN
1
2
3
4
5
[GALACTOSE-6P (mM)]

Figure 17. Gas-liquid chromatographic separation of the trimethylsilylated products of rat liver microsomal and rat brain soluble $NADP^+$-dependent galactose 6-phosphate dehydrogenase activities.

The reaction mixtures were prepared for gas-liquid chromatography as described in Materials and Methods. The separation of the reactants and products prior to the incubation period, with and without alkaline phosphatase treatment, are shown in tracings A and C, respectively. Tracings B and D show the separation of the reactants and products after the incubation period, with and without alkaline phosphatase treatment. Peaks 1, 2, and 3 are γ, α, and β-D-galactose (30), respectively. Peak 4 is an unknown and peak 5 is D-galactonate.

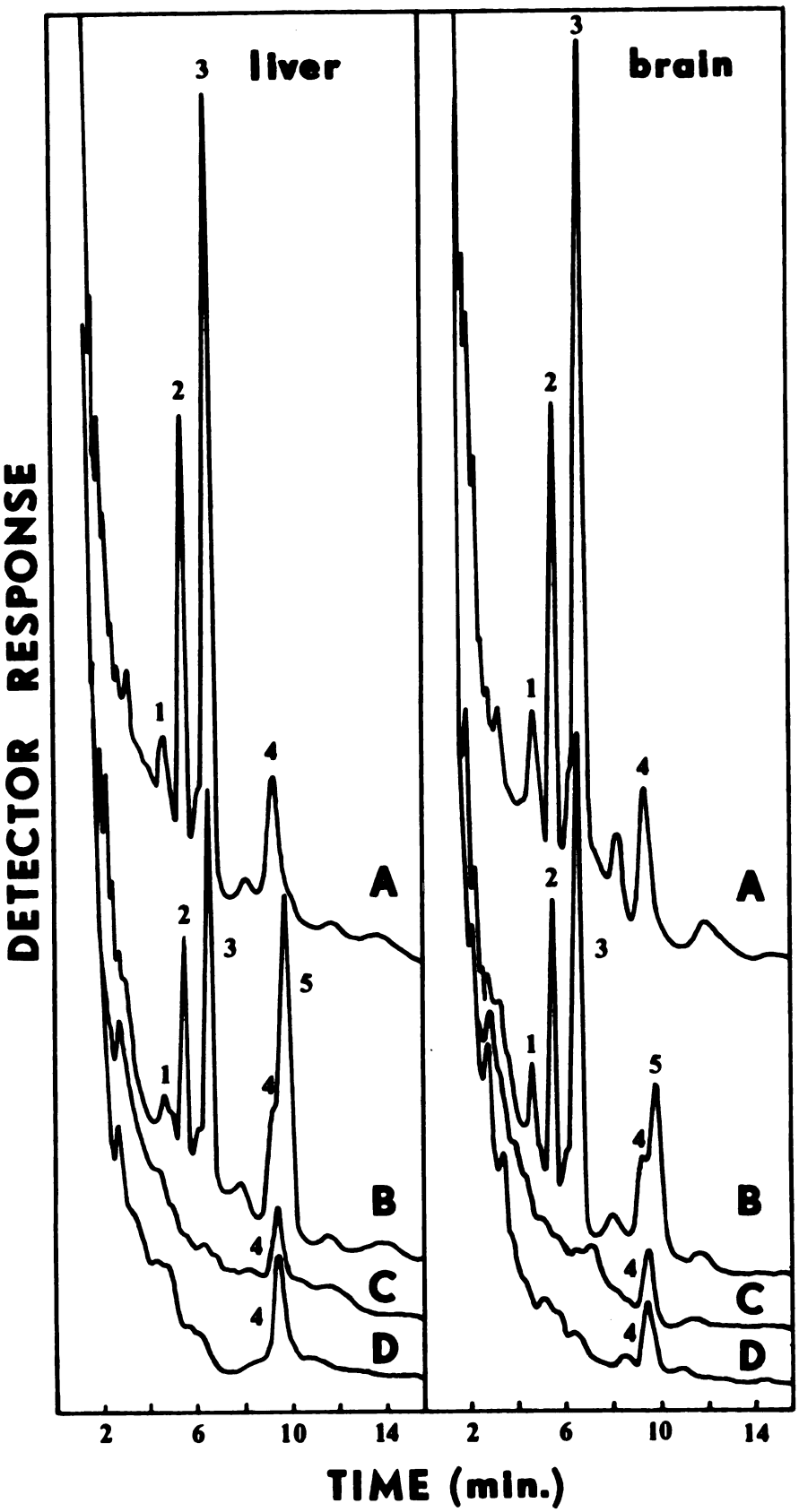

liver
brain
DETECTOR RESPONSE
TIME (min.)
A
B
C
D
1
2
3
4
5
2
6
10
14

Figure 18. Lineweaver-Burk plots of rat brain of galactose 6-phosphate (□——□, ●——●), and galactose 1-phosphate (0——0) phosphatase activities.

Assay methods are described under Materials and Methods. Velocities are in nmoles galactose released/min/gm fresh tissue.

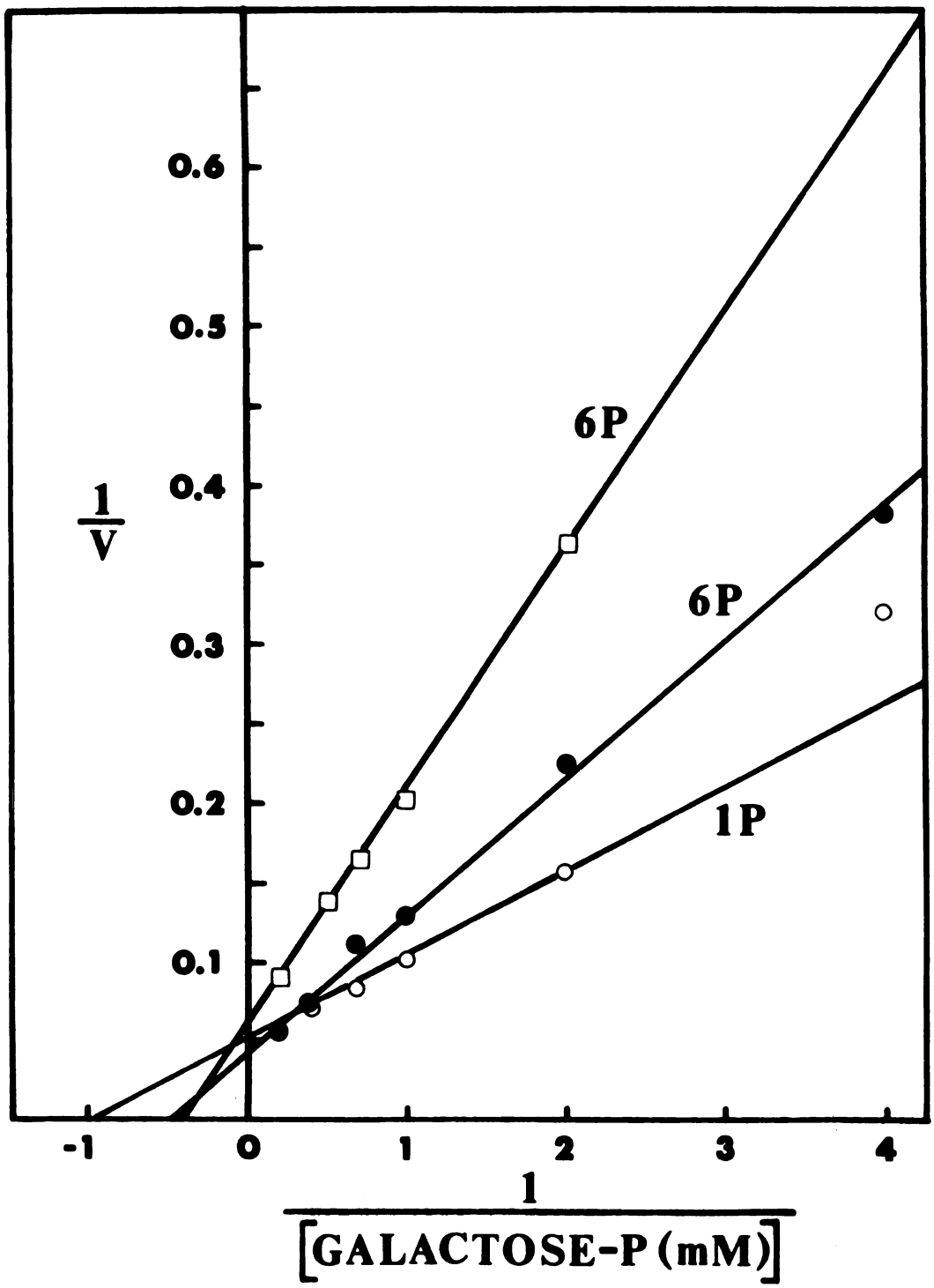

0.6
0.5
0.4
0.3
0.2
0.1
1/V
6P
6P
1P
-1
0
1
2
3
4
1
[GALACTOSE-P (mM)]

Figure 19. Rat brain 6-phosphogalactonate phosphatase activity as a function of 6-phosphogalactonate concentration.

The assay method is described under Materials and Methods. The reaction velocity is in nmoles phosphate released/min/gm fresh tissue.

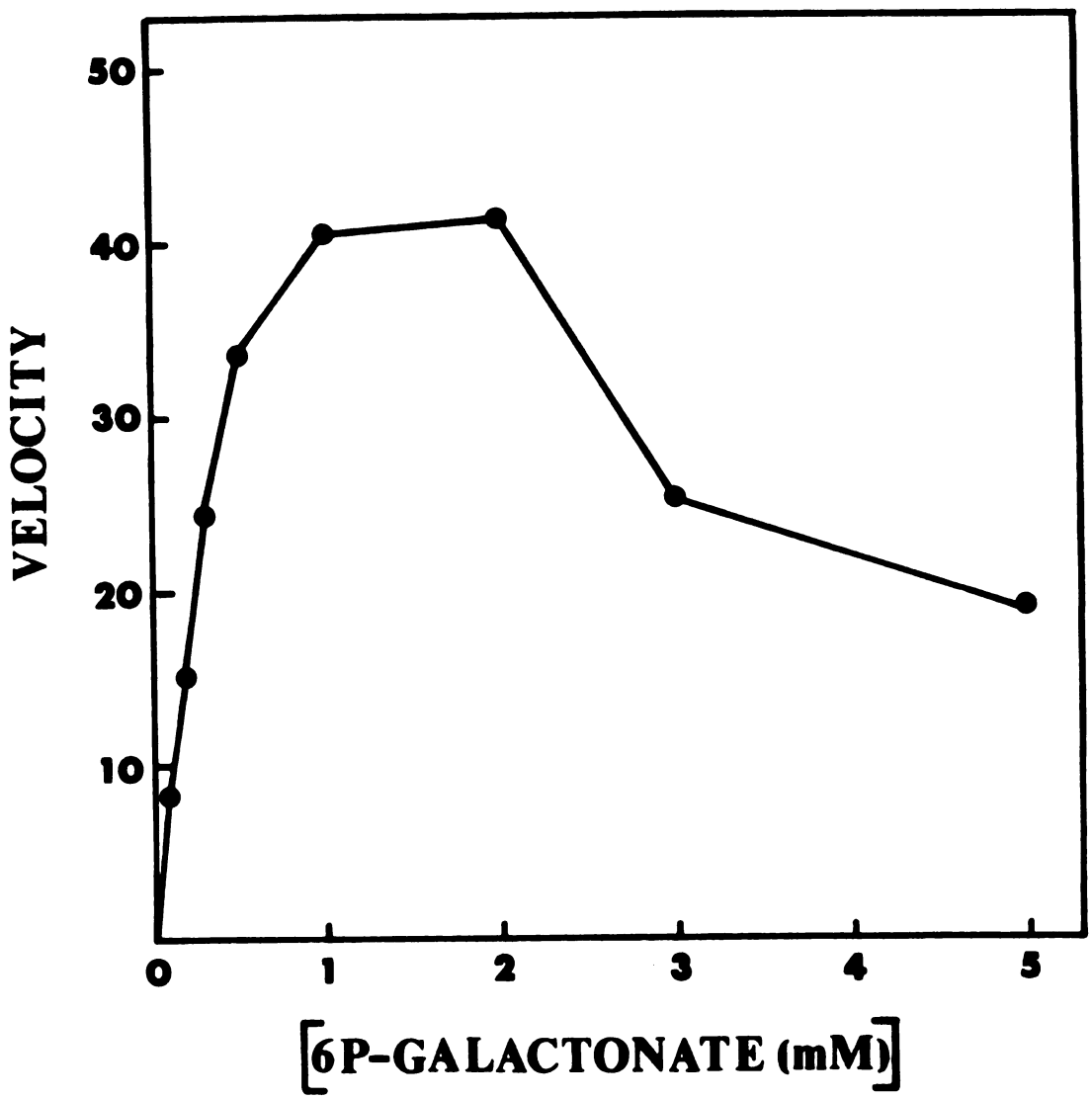

VELOCITY
50
40
30
20
10
0
1
2
3
4
5
[6P-GALACTONATE (mM)]

Figure 20. Effect of galactose 6-phosphate and 6-phosphogalactonate on rat brain phospho-glucose isomerase.

A substrate-velocity plot of brain phospho-glucose isomerase activity (●——●) in the presence of galactose 6-phosphate (0.2 mM, 0——0 and 1.0 mM, ▲——▲) and 6-phosphogalactonate (0.4 mM, □——□). Velocity is given in μmoles fructose 6-phosphate formed from glucose 6-phosphate/min/gm fresh tissue.

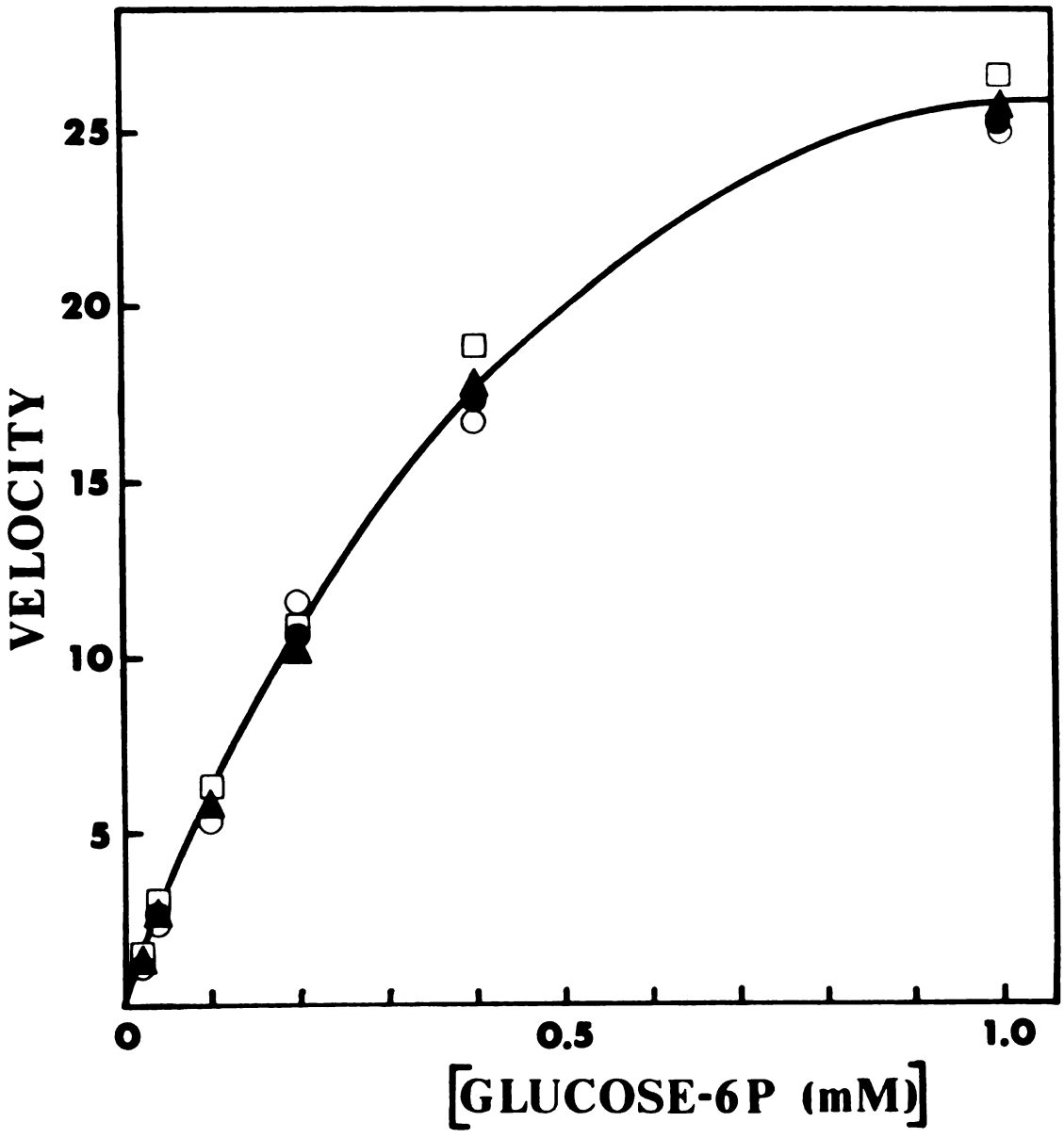

VELOCITY
25
20
15
10
5
0
0.5
1.0
[GLUCOSE-6P (mM)]

Figure 21. Effect of galactose 6-phosphate and 6-phosphogalactonate on rat brain glucose 6-phosphate dehydrogenase.

A substrate-velocity plot of brain glucose 6-phosphate dehydrogenase activity (0——0) in the presence of galactose 6-phosphate (0.2 mM, ●——● and 1.0 mM, □——□) and 6-phosphogalactonate (0.4 mM, ▲——▲). Velocity is given in μmoles glucose 6-phosphate oxidized/min/gm fresh tissue.

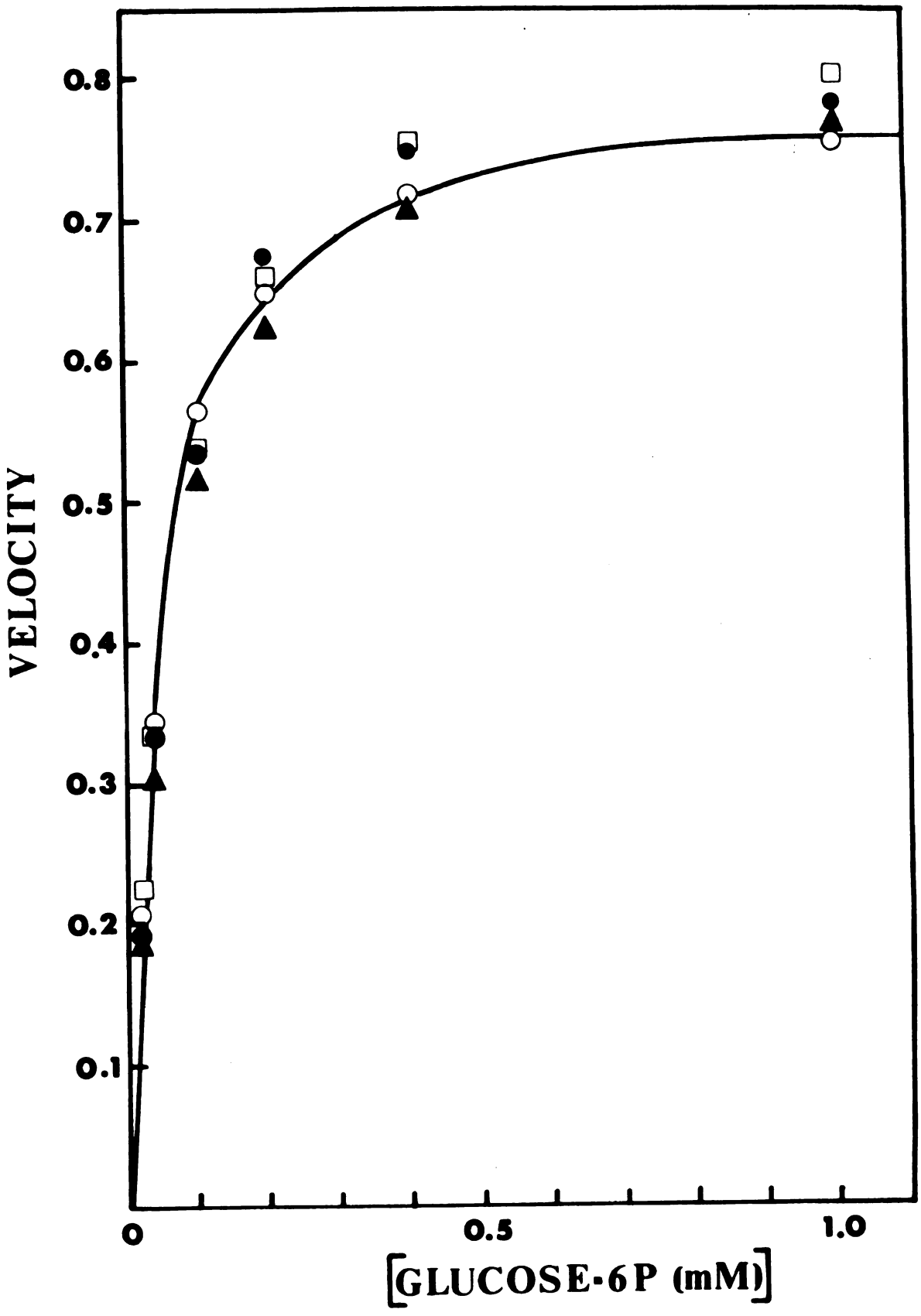

VELOCITY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5
1.0
[GLUCOSE-6P (mM)]

Figure 22. Effect of galactose 6-phosphate on rat brain soluble hexokinase.

A substrate velocity plot of soluble brain hexokinase (●——●) in the presence of galactose 6-phosphate (0.2 mM, ▲——▲ and 1.0 mM, □——□). Velocity is given in μmoles glucose phosphorylated/min/gm fresh tissue.

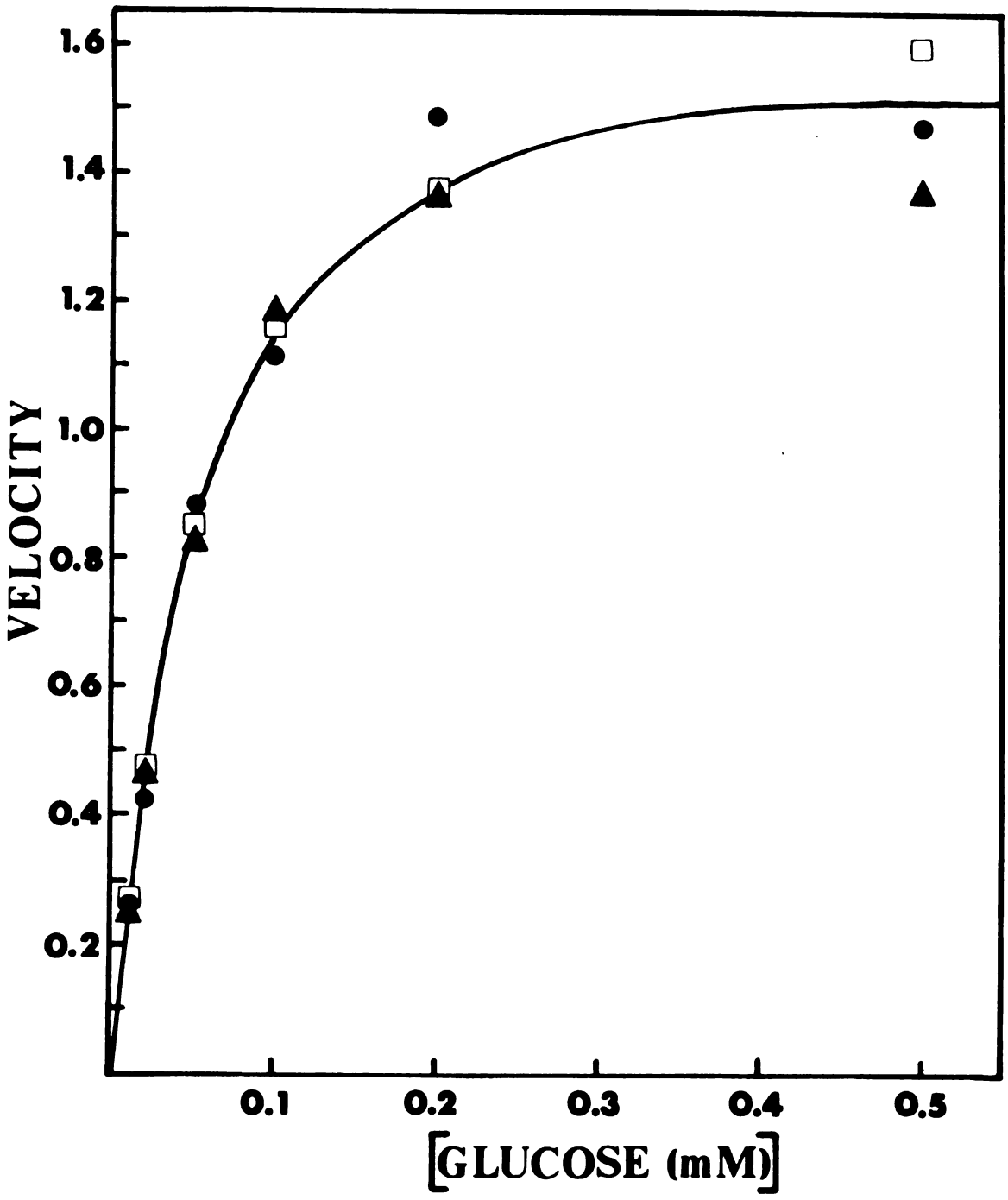

VELOCITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.1
0.2
0.3
0.4
0.5
[GLUCOSE (mM)]

Figure 23. The effect of 6-phosphogalactonate on rat brain 6-phosphogluconate dehydrogenase.

A Lineweaver-Burk plot of 6-phosphogluconate dehydrogenase activity in the presence of increasing amounts of 6-phosphogalactonate. Assay methods are described in Materials and Methods. Velocities are given in μmoles 6-phosphogluconate oxidized/min/gm fresh tissue.

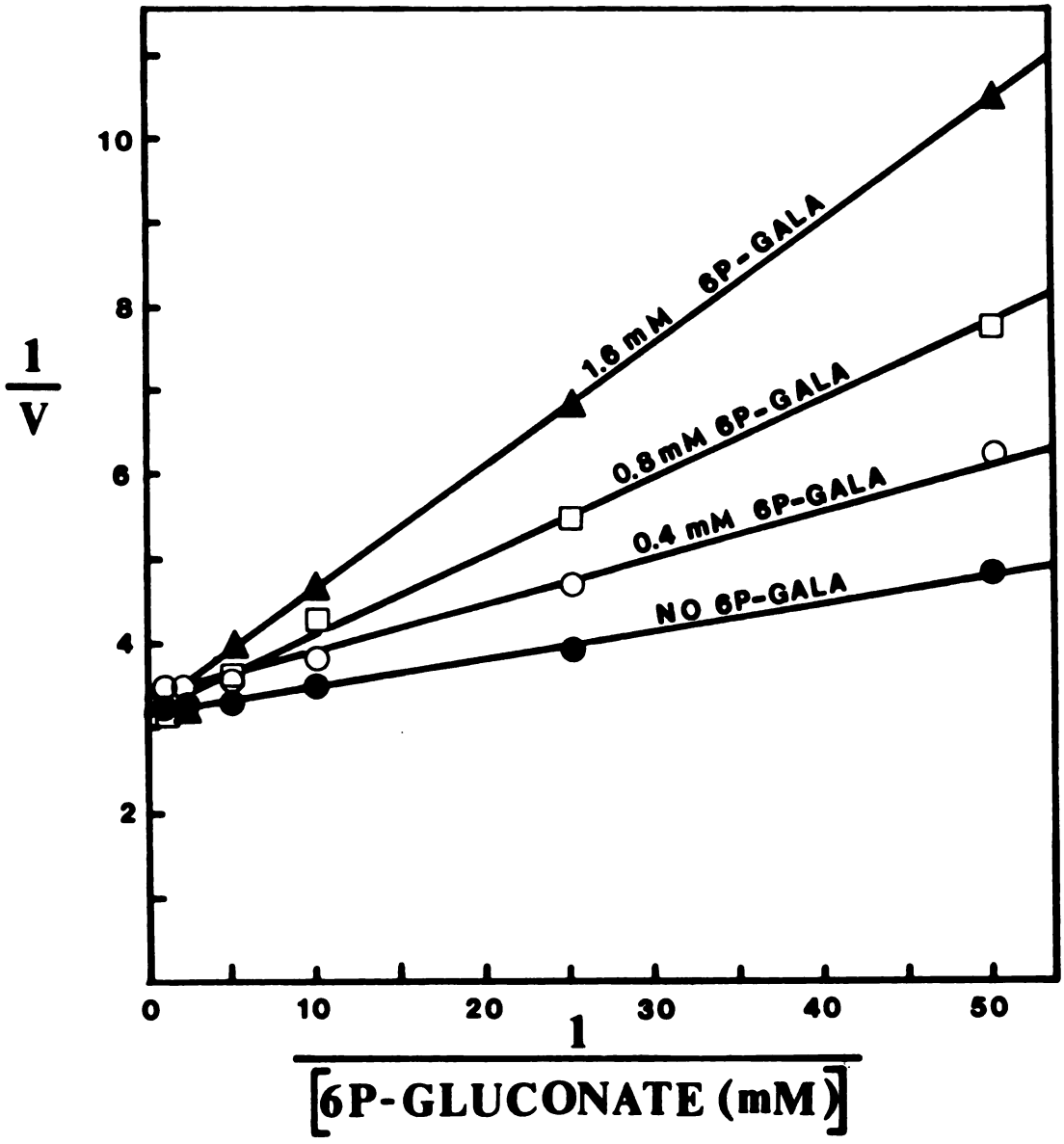
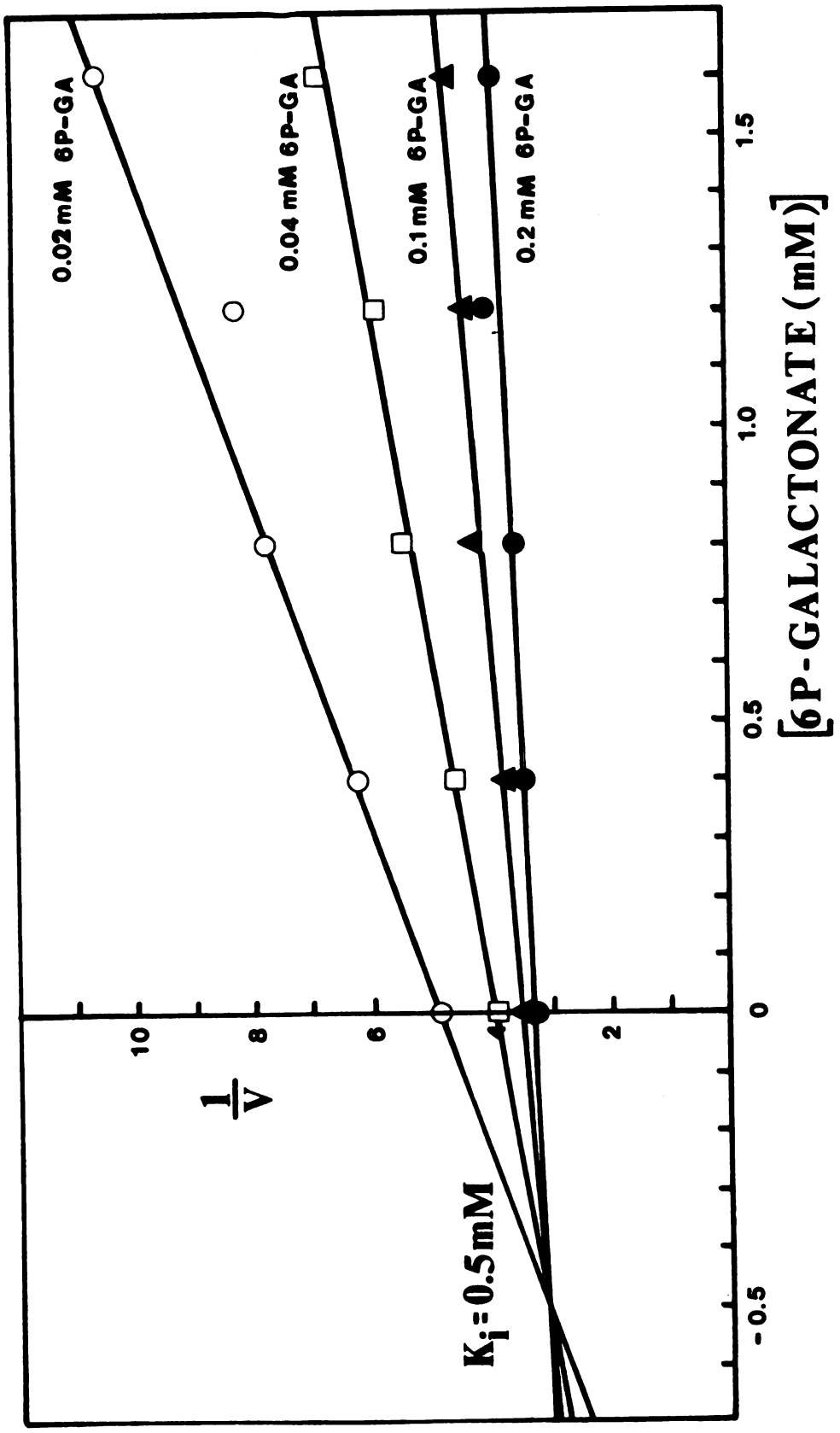

1.6 mM 6P-GALA
0.8 mM 6P-GALA
0.4 mM 6P-GALA
NO 6P-GALA
1/V
1/[6P-GLUCONATE (mM)]
0
10
20
30
40
50
2
4
6
8
10

Figure 24. Dixon plot for the Ki determination of 6-phosphogalactonate for rat brain 6-phosphogluconate dehydrogenase.

Assay methods are described under Materials and Methods. Velocities are given in μmoles 6-phosphogluconate oxidized/min/gm fresh tissue.


0.02 mM 6P-GA
0.04 mM 6P-GA
0.1 mM 6P-GA
0.2 mM 6P-GA
1/V
10
8
6
4
2
0
-0.5
0.5
1.0
1.5
[6P-GALACTONATE (mM)]
Ki=0.5mM

SUMMARY

SUMMARY

The question asked at the outset of this work was whether the isolated perfused rat heart was a suitable model for studying classic galactosemia. Since earlier reports had suggested that D-galactose was not actively metabolized by rat heart, studies were undertaken to determine the ability of heart slices to oxidize galactose to carbon dioxide. For comparative purposes, glucose was also examined as an oxidative substrate. Galactose was found to be oxidized to carbon dioxide at a rate no more than 5% that of glucose oxidation, indicating that galactose was not appreciably utilized for metabolic energy.

To determine the role of the reductive pathway in rat heart, isolated hearts were perfused with varying amounts of galactose for a fixed time period. The tissue concentration of galactitol was found to be directly related to the amount of galactose in the perfusing medium and it also appeared to be substrate saturable. A consideration of the kinetics of galactitol production in the perfused heart suggested that galactitol was being synthesized by aldose reductase. Furthermore, a comparison of galactose oxidation by heart slices with galactitol synthesis in the perfused heart suggested that more galactose is reduced than oxidized at galactose

levels above 3 mM.

The possibility of a block in the enzymatic conversion of galactose to glycolytic metabolites was investigated as an explanation for the poor oxidation of galactose by heart. Accordingly, the ability of heart and liver homogenates to synthesize intermediates of the Leloir pathway were compared. During the incubation period, liver homogenates readily converted galactose to galactose phosphate, but heart homogenates did not produce any detectable phosphorylated metabolites. This suggested that galactose phosphorylation was rate limiting in the cardiac utilization of galactose. A subsequent analysis of the Leloir pathway enzymes in heart showed that galactokinase was indeed the lowest in activity of the three enzymes. These observations support the contention that galactose phosphorylation is rate limiting in rat heart galactose catabolism. On the other hand, the distribution of the Leloir enzymes in liver and brain were quite different from heart. The lowest activity of the three enzymes in brain and liver were uridyl transferase and UDP-galactose epimerase, respectively. Therefore, since galactose 1-phosphate uridyl transferase was not rate limiting in cardiac tissue, heart does not appear to be a suitable model for studying classic galactosemia. However, the rat myocardium might be useful in the study of certain factors believed to be pathogenic in classic galactosemia. For example, heart accumulates large amounts of galactitol, and galactitol accumulation has been implicated in the disruption of lysosomal integrity in

nervous tissue. Therefore, in an isolated perfused heart system, tissue accumulation of galactitol might be correlated with the release of lysosomal hydrolases into the perfusion medium. Another pathologic mechanism believed to be operating in uridyl transferase deficiency is the inhibition of glucose entry into the cell by galactose. The competitive inhibition of glucose transport by galactose in heart tissue has been related to a depression in certain glycolytic metabolites, and this report has presented preliminary evidence that heart ATP and creatine phosphate levels are reduced by galactose perfusion. Since cardiac tissue can maintain normal energy metabolite levels in the absence of glucose when fatty acids or ketone bodies are provided, heart should be useful in studying the effects of galactose metabolites on cellular metabolism separated from the inhibition of glucose transport by galactose.

The second portion of this work was concerned with the identification and metabolism of galactose 6-phosphate in heart and brain. Initial experiments suggested that galactose 6-phosphate was produced at elevated galactose levels by rat heart and brain soluble fractions. To determine if this metabolite could be synthesized in vivo, two systems in which high galactose levels are attainable were examined. Rat hearts perfused with high concentrations of galactose and newborn chicks fed large amounts of galactose were found to contain galactose 6-phosphate at tissue concentrations similar to those of certain glycolytic metabolites but only

comprising about 5% of the total tissue galactose-phosphate.

Since both galactokinase and uridyl transferase deficient galactosemics have a residual capacity to oxidize galactose and are able to oxidize C-1 of galactose faster than C-2, a direct oxidative pathway leading to pentose has been postulated to be operating in these diseases. While such a direct pathway is possible, a galactose 6-phosphate oxidative pathway is also consistent with the above observations. Accordingly, the metabolism of galactose 6-phosphate was investigated in rat heart and brain. An $NADP^+$-dependent galactose 6-phosphate dehydrogenase was found in brain soluble fractions which appeared to be distinct from the liver microsomal activity. However, in light of the low tissue levels of galactose 6-phosphate and the unfavorable Km of the rat brain enzyme, oxidation of galactose 6-phosphate to 6-phosphogalactonate is unlikely. Furthermore, since 6-phosphogalactonate was not oxidized by rat brain subcellular fractions, the metabolism of galactose 6-phosphate to pentose apparently does not occur in brain. If 6-phosphogalactonate were to form in brain, it could be hydrolyzed by an alkaline phosphatase activity. While a galactose 6-phosphate phosphatase activity was only found in rat brain, it is possible that other phosphatases could be operating in heart and brain over different pH ranges.

The concentrations of blood galactose found in cases of classic galactosemia is several times higher than in galactokinase deficiency although mental retardation is associated

only with the former. Since the formation of galactose 6-phosphate appeared to be a result of high galactose levels in heart and brain, galactose 6-phosphate was investigated as a potentially toxic metabolite which might contribute to the more severe symptomology of classic galactosemia. Because of the structural similarity with the glucose analogues, galactose 6-phosphate and 6-phosphogalactonate were tested as inhibitors of several glycolytic and hexose monophosphate shunt enzymes. Since glucose 6-phosphate and galactose 6-phosphate were found at comparable tissue concentrations in heart and brain, it was reasonable to suspect that some degree of inhibition might occur physiologically. However, none of the enzymes tested were affected at galactose 6-phosphate levels as high as fifty fold in excess of the substrate levels. Only 6-phosphogluconate dehydrogenase was inhibited by 6-phosphogalactonate, but the high Ki suggests that the enzymes would not be affected by 6-phosphogalactonate if it were to form in vivo. Summarizing, it therefore appears that a viable pathway of galactose 6-phosphate metabolism in brain and heart does not occur. Furthermore, it is unlikely that galactose 6-phosphate or 6-phosphogalactonate are factors in the pathogenic mechanisms of classic galactosemia.

APPENDIX

APPENDIX

Hydrolysis of Galactose 1-Phosphate and Galactose 6-Phosphate in 10% Trichloroacetic Acid

Aliquots [100 µl] of 20 mM galactose 1-phosphate and galactose 6-phosphate were placed in small culture tubes containing either 5 µl water or 5 µl alkaline phosphatase [10 mg/ml, Worhtington]. The set of four tubes were incubated at 25°C for 3 hours. Following the incubation period 25 µl of each tube were placed in large culture tubes containing 2.5 ml of 10% TCA. The tubes were covered with marbles and heated in a boiling water bath for 20 minutes.

At 0, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 15 and 20 minutes, 0.2 ml of each hydrolysis tube was removed for inorganic phosphate analysis [see Chapter II]. As can be seen in Figure 25, hydrolysis of galactose 1-phosphate is virtually complete by 1 minute. On the other hand, galactose 6-phosphate is stable over the 20 minute hydrolysis time. For comparison, samples pretreated with alkaline phosphatase are also shown in Figure 25.

Hydrolysis of Tissue Galactose 1-Phosphate

Two grams of pulverized chick brain and rat heart were homogenized in 10% TCA as described in Chapter II. Prior to the first centrifugation, 1 µmole of [U-^{14}C]-D-galactose

1-phosphate [0.2 μCi] was added to the homogenates. The extraction and hydrolysis procedure in Chapter II was followed through the paper chromatographic separation. The regions corresponding to the sugar monophosphates and neutral sugars were cut out and counted in 10 ml Bray's solution [Chapter I]. The results, corrected for background CPM , are shown in Table 6. As can be seen, greater than 99.3 % of the galactose 1-phosphate is hydrolyzed.

Recovery of [U-^{14}C] Glucose 6-Phosphate from TCA-Extracted Tissue

Two 5 gm samples of pulverized chick brain and rat heart were homogenized in 10% TCA as described in Chapter II. Prior to the first centrifugation, 80 nmoles [0.25 μCi] of [U-^{14}C]-D-glucose 6-phosphate were added to the homogenates. The %-recovery during several steps of the extraction procedure are shown in Table 7. The average recovery for the complete process is about 30%, with the largest losses occuring during ether and paper extractions.

Characterization of Synthetic 6-Phospho-D-Galactonic Acid

Assuming a molecular weight of 411.4, a 20 mM aqueous solution of synthetic barium D-6-phosphogalactonate was prepared and exchanged with potassium. An 0.91 ml aliquot of this solution was made to a 1.0 ml total volume with 20 μl 1.0 M Tris-HCl, pH 10.0, 20 μl of Sigma alkaline phosphatase [see Chapter II] and 50 μl of water. The solution was incubated at 37°C for 270 minutes. At 0, 1, 5, 10, 30, 60, 90, 150, 210 and 270 minutes, 50 μl aliquots of the

reaction mixture were removed and analyzed for inorganic phosphate, galactonate and galactonolactone [see Chapter II]. Figure 26 shows the release of galactonate plus galactonolactone and inorganic phosphate with time. Since 0.91 µmoles of 6-phosphogalactonate should have been found per 50 µl aliquot and an end point of 0.515 µmoles (Figure 26) was found, the synthetic compound is only about 57% pure [by weight]. Furthermore, since a 1:1 stoichiometry is found between inorganic phosphate and the sum of galactonolactone and galactonate, the impurities are considered to be $BaCO_3$. A GLC tracing [190°C oven temperature] of alkaline phosphotase treated and not treated 6-phosphogalactonate is shown in Figure 27, A and B respectively. Peaks 1, 2, 3 and 4 correspond to α-methyl-mannoside [standard], galactono-γ-lactone, galactonate and 6-phosphogalactonate respectively. As can be seen, alkaline phosphotase treatment releases only galactonolactone and galactonate from 6-phosphogalactonate. For comparative purposes, four GLC tracings [225°C oven temperature] of 6-phosphogluconate, synthetic 6-phosphogalactonate and galactose 6-phosphate are shown in Figure 28, frames A, B and C respectively. The retention time of 6-phosphogalactonate is identical with α-D-galactopyranose 6-phosphate (peak 3) and slightly slower than 6-phosphogluconate (peak 1).

Gas Chromatographic Separation of Galactose, Galactonate and Galactonolactone

The sodium salt of galactonic acid was prepared by treating galactono-γ-lactone with 0.1 M NaOH. Standards of galactonolactone and galactose were prepared in 0.1 M HCl and water respectively. Samples were dried by rotary evaporation and trimethylsilylated as described in Chapter II. The GLC tracings [188°C oven temperature] of galactonate (A), galactonolactone (B) and galactose (C) are shown in Figure 29. Peaks 1, 2, 3, 4 and 5 correspond to galactonolactone, galactonate, α-D-galactofuranose, α-D-galactopyranose and β-D-galactopyranose respectively. As can be seen, galactonolactone has the same retention time as α-D-galactopyranose. Galactonate, on the other hand is clearly separated from all isomers of galactose.

Table 6. Hydrolysis of Tissue Galactose 6-Phosphate.

Sample	Sugar Monophosphate CPM	Neutral Sugar CPM	% Total in Sugar Monophosphate
Brain	119	16,040	0.7%
Heart	146	18,634	0.7%

Table 7. Recovery of [U-^{14}C] Glucose 6-Phosphate from TCA Extracted Tissue.

Step	Sample	%-Recovery
Total TCA extraction (Prior to Boiling)	B-1	
	B-2	107.4
	H-1	101.4
	H-2	100.3
Neutralized and Concentrated Ether Extracts	B-1	61.6
	B-2	64.1
	H-1	69.9
	H-2	66.3
Final Concentrated Paper Eluates	B-1	28.9
	B-2	31.0
	H-1	28.8
	H-2	33.0

B and H refer to brain and heart tissue preparations.

Figure 25. Hydrolysis of galactose 1-phosphate and galactose 6-phosphate in 10% trichloroacetic acid.

Symbols represent: galactose 1-phosphate (○——○); alkaline phosphotase treated galactose 1-phosphate (●——●); galactose 6-phosphate (▲——▲); alkaline phosphotase treated galactose 6-phosphate (□——□). Experimental conditions are described in the Appendix.

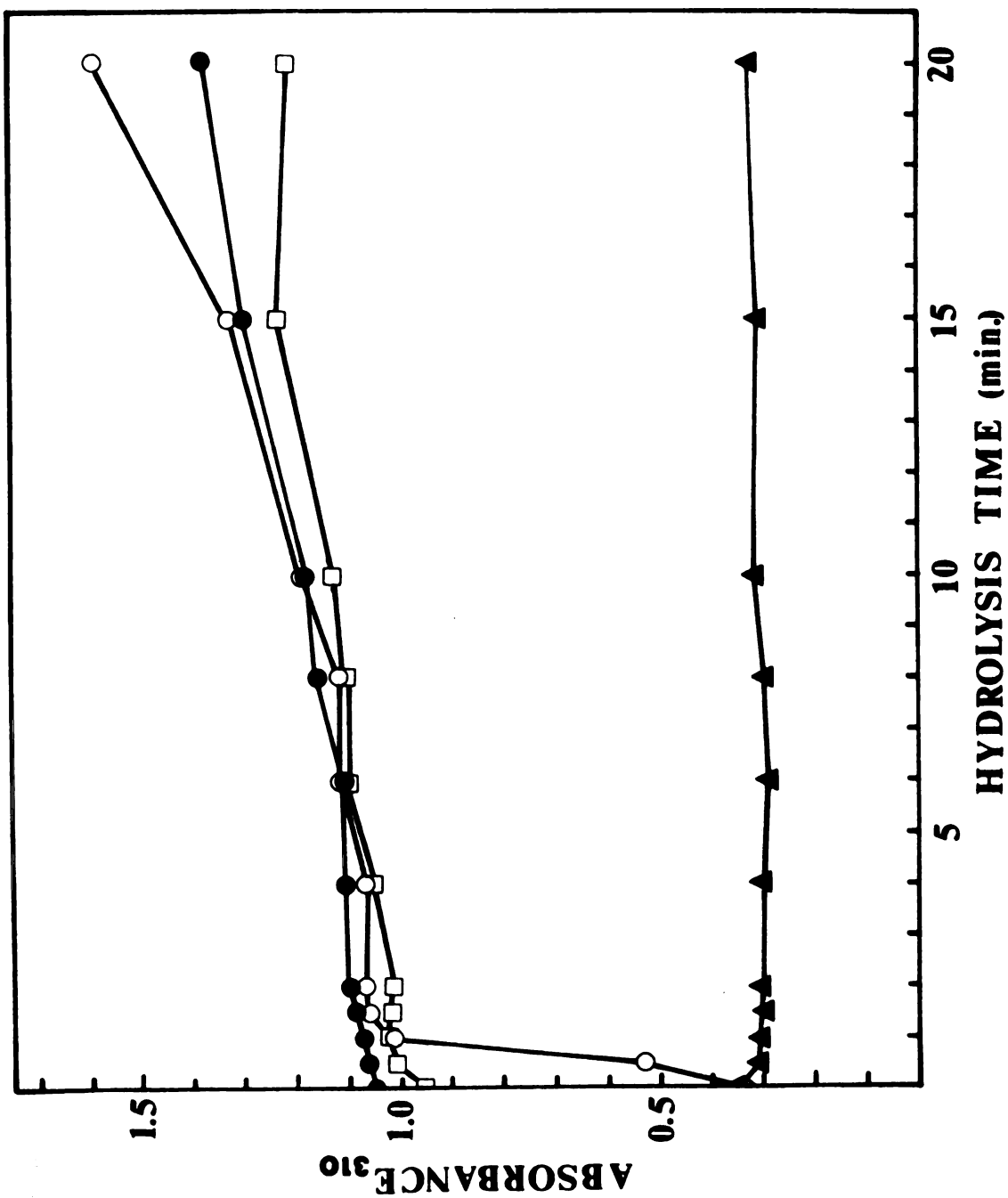

ABSORBANCE 310
1.5
1.0
0.5
5
10
15
20
HYDROLYSIS TIME (min.)

Figure 26. Stoichiometry between the sum of galactonate and galactonolactone and inorganic phosphate released from alkaline phosphotase treated 6-phosphogalactonate.

Symbols represent: inorganic phosphate (●——●); the sum of galactonate and galactonolactone (0——0). Experimental conditions are described in the Appendix.

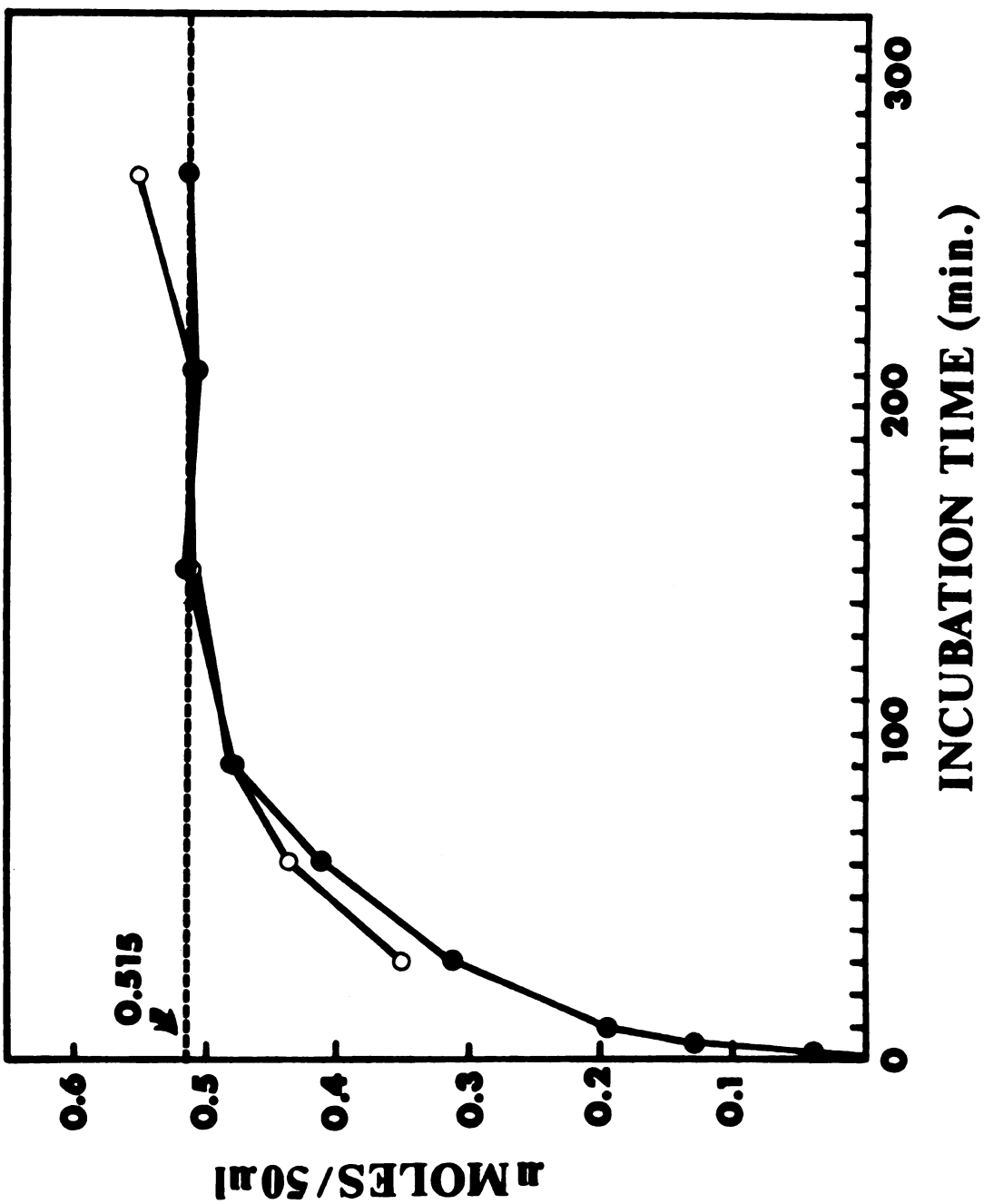

0.515
0.6
0.5
0.4
0.3
0.2
0.1
μMOLES/50μl
0
100
200
300
INCUBATION TIME (min.)

Figure 27. Gas liquid chromatography of 6-phosphogalactonate and alkaline phosphatase treated 6-phosphogalactonate.

In Frame A peaks represent: (1) α-methyl-mannoside [standard]; (2) galactono-γ-lactone; (3) galactonate. In Frame B peaks are: (1) α-methyl-mannoside [standard]; (4) 6-phosphogalactonic acid. Experimental conditions are described in the Appendix.

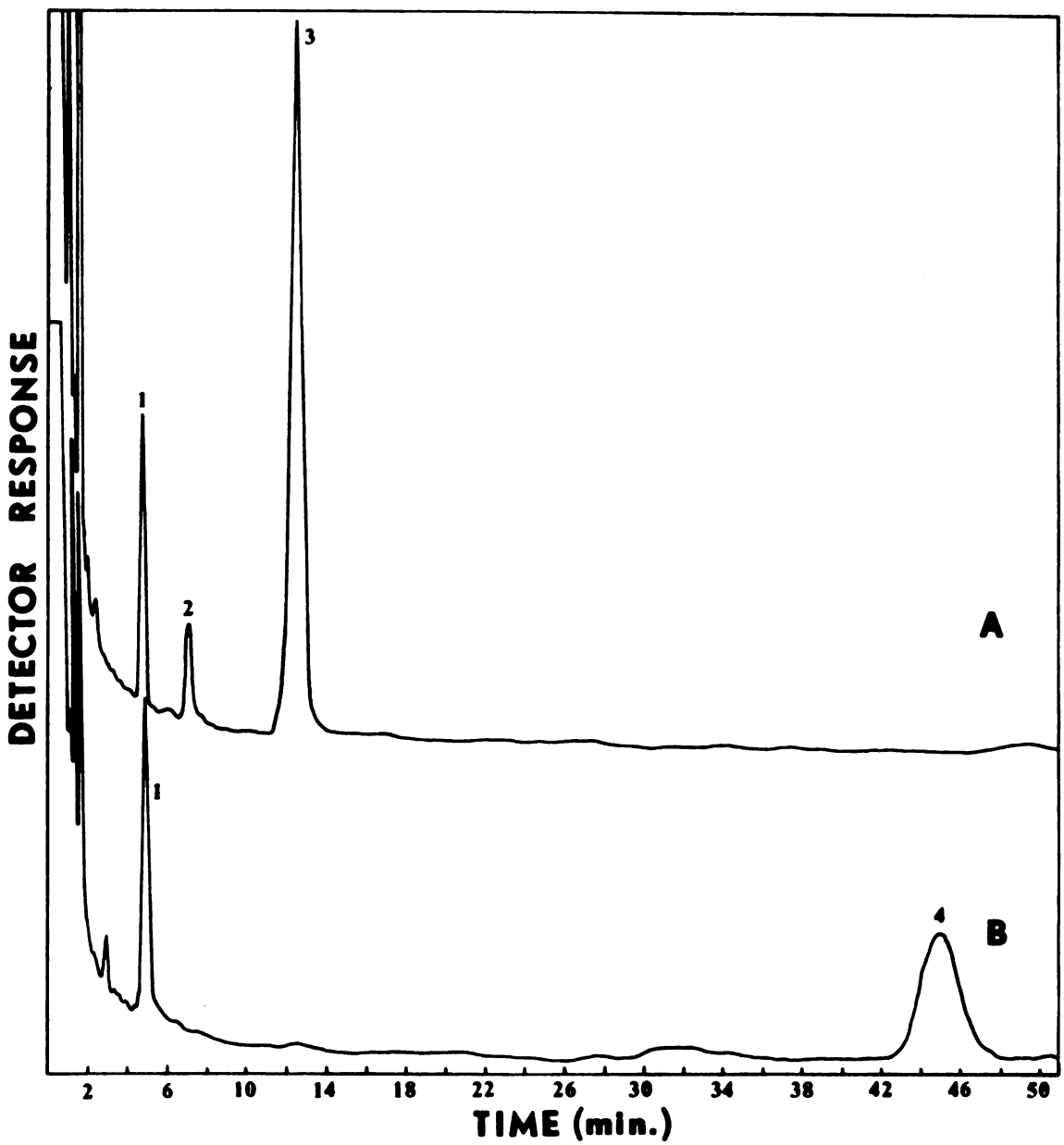

DETECTOR RESPONSE
1
2
3
A
1
4
B
2
6
10
14
18
22
26
30
34
38
42
46
50
TIME (min.)

Figure 28. Gas liquid chromatography of 6-phosphogluconate, 6-phosphogalactonate and galactose 6-phosphate.

Peaks in Frame 1 represent: (1) 6-phosphogluconate and (2) 6-phosphogluconolactone. Peaks in Frame 2 represent: (1) 6-phosphogalactonate. Peaks in Frame 3 represent: (1) and (2) α and β-D-galactofuranose 6-phosphate and (3) and (4) α and β-D-galactopyranose 6-phosphate. Experimental conditions are described in the Appendix.

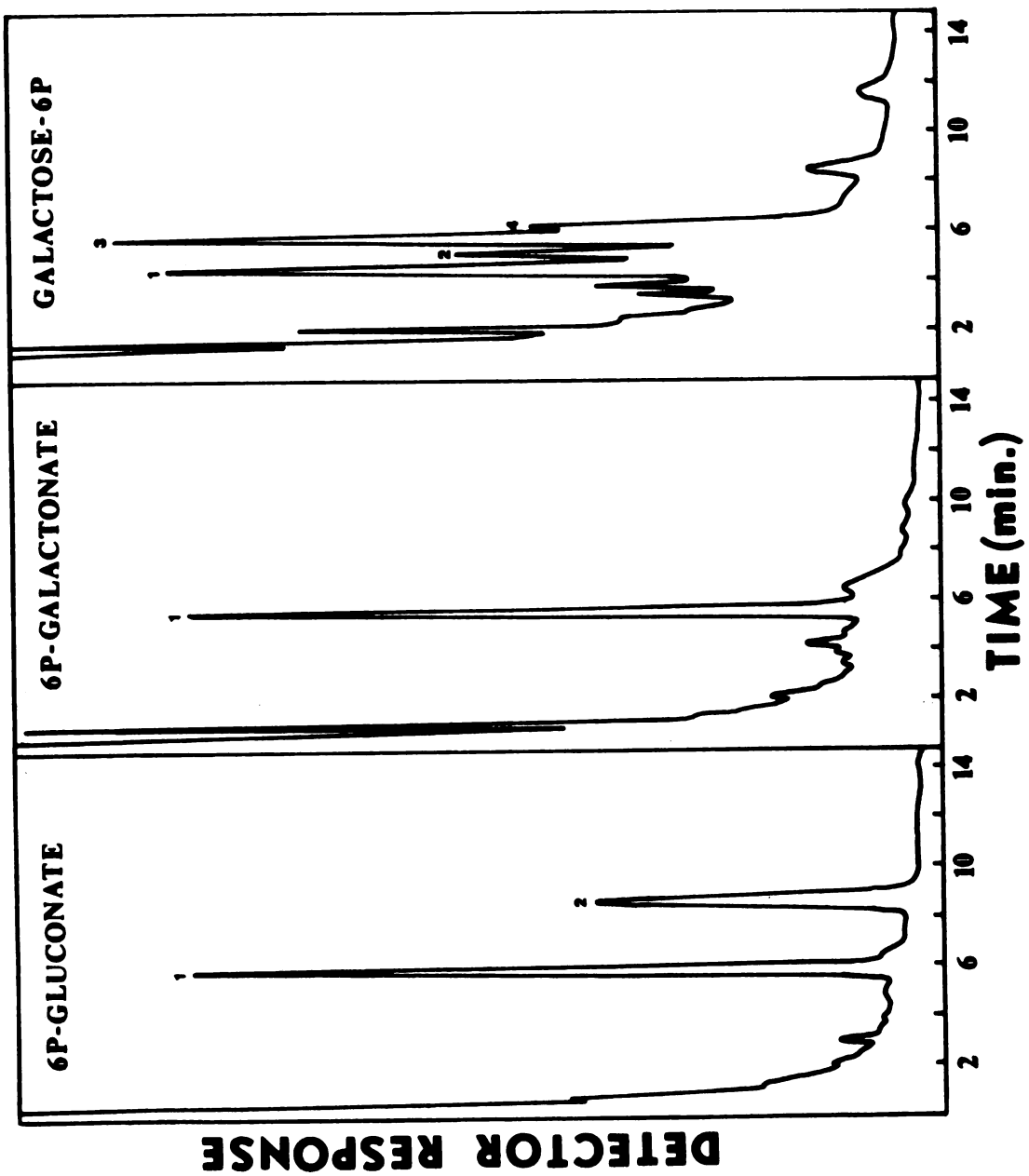
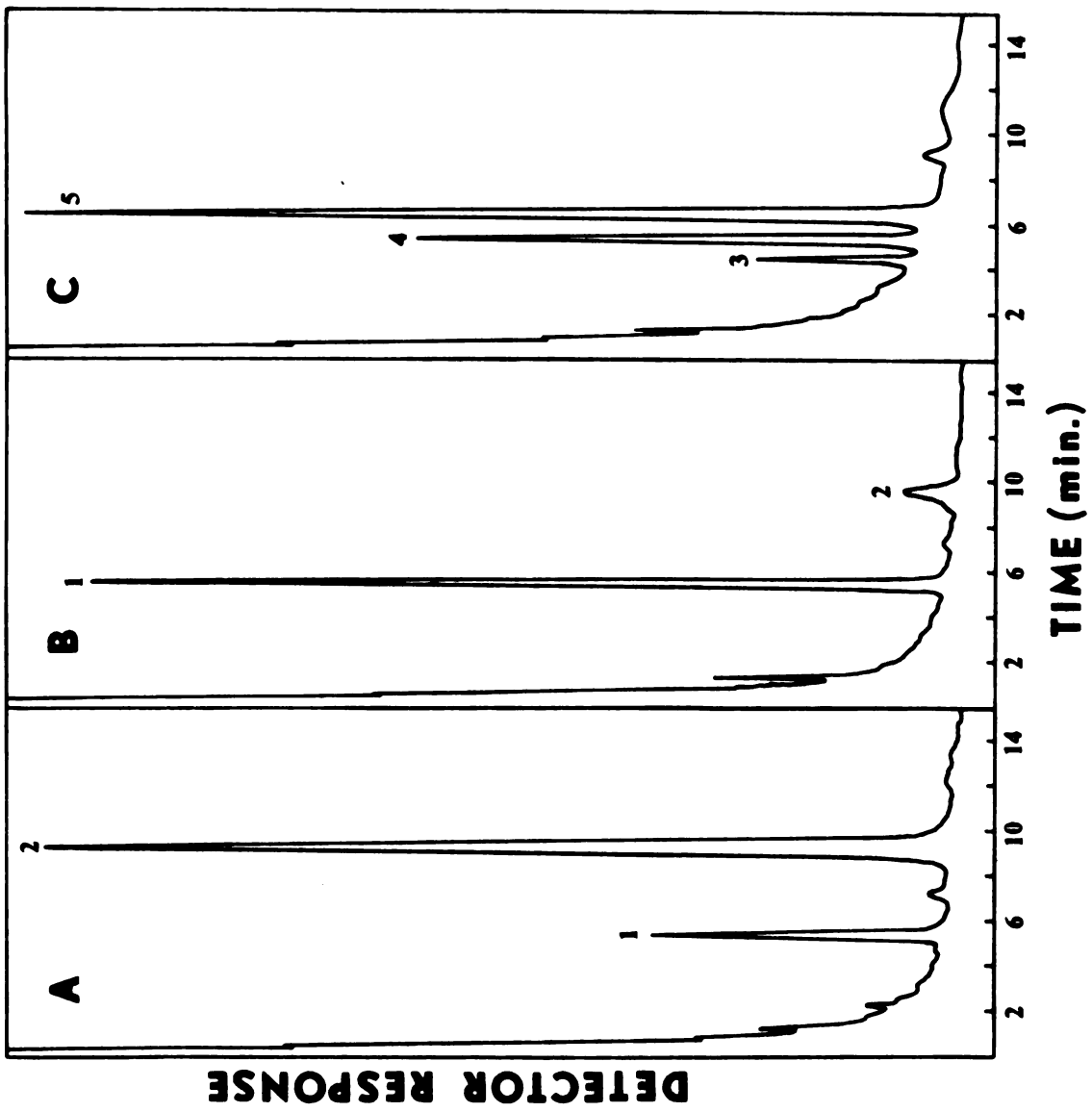

6P-GLUCONATE
6P-GALACTONATE
GALACTOSE-6P
DETECTOR RESPONSE
TIME (min.)
2
6
10
14

Figure 29. Gas liquid chromatography of galactonate, galactonolactone and galactose.

In Frames A and B peaks represent: (1) galactono-γ-lactone and (2) galactonate. In Frame C peaks are: (3) α-D-galactofuranose, (4) and (5) α and β-D-galactopyranose respectively. Experimental conditions are described in the Appendix.


A
B
C
1
2
3
4
5
2
6
10
14
TIME (min.)
DETECTOR RESPONSE