INBORN ERRORS OF METABOLISM

I. THE PRESENCE OF AN INACTIVE ENZYME AND *IN UTERO* TOXICITY IN GALACTOSEMIA

II. URINARY MUCOPOLYSACCHARIDES IN PATIENTS WITH HURLER'S SYNDROME, THEIR FAMILIES AND NORMAL MAN

Thesis for the Degree of Ph. D.

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Jary S. Mayes

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thesis entitled

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I. The Presence of an Inactive Enzyme and *in utero* Toxicity in Galactosemia

II. Urinary Mucopolysaccharides in Patients with Hurler's Syndrome, Their Families and Normal Men

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ABSTRACT

INBORN ERRORS OF METABOLISM

I. THE PRESENCE OF AN INACTIVE ENZYME AND IN UTERO TOXICITY IN GALACTOSEMIA

II. URINARY MUCOPOLYSACCHARIDES IN PATIENTS WITH HURLER'S SYNDROME, THEIR FAMILIES AND NORMAL MAN

by Jary S. Mayes

PART I

Galactosemia is a disorder of galactose metabolism in humans in which Gal-1-P uridyl transferase activity is very low or completely absent in these individuals. Experiments were designed to determine if the protein is completely lacking or altered to an inactive form.

Gal-1-P uridyl transferase was purified about 500 fold over a crude extract from calf liver and some of its properties were studied. Antibody against the purified protein was prepared by injecting it into rabbits. The serum from these rabbits precipitates the calf liver and human erythrocyte enzymes. A fraction from the blood of patients with galactosemia precipitates the antibody suggesting an inactive Gal-1-P uridyl transferase protein in galactosemia.

The heterozygous carriers of galactosemia are usually asymptomatic. However, two children were observed who had

some of the symptoms of galactosemia, but the transferase levels in the erythrocytes were in the heterozygous range. In the mothers of these individuals, transferase levels were in the low heterozygous range suggesting that *in utero* toxicity could have occurred during pregnancy.

PART II

Hurler's syndrome is a disorder of mucopolysaccharide metabolism in which chondroitin sulfate B and/or heparitin sulfate are excreted in the urine and accumulate in the tissues. The heterozygotes do not show any symptoms, but small abnormal amounts of mucopolysaccharides may be excreted in the urine. It is also important to know the normal types and amounts of mucopolysaccharides when studying mucopolysaccharide disorders.

Therefore, it was of interest to measure the mucopolysaccharides in urine samples from Hurler's syndrome, their families and normal man. A marked decrease in the amount of total mucopolysaccharides and chondroitin sulfates per unit of creatinine was observed with decreasing age in normal children, but the amount became constant with age after the late teens. In the urine samples from Hurler's syndromes, abnormal amounts of heparitin sulfate were found in five patients and abnormal amounts of heparitin sulfate and chondroitin sulfate B were found in one patient. It is

suggested that the type of Hurler's syndrome that excretes only heparitin sulfate may be more common than previously thought. It was possible to obtain urine samples from some of the families of these patients. The mucopoly-saccharides in these samples were within the normal range by all criteria employed.

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To Faye

VITA

Jary S. Mayes was born on October 19, 1938 at Walters, Oklahoma. He graduated from Walters High School in 1956. After attending Cameron Junior College, he transferred to Oklahoma State University and received the Bachelor of Science degree in 1960 from the Department of Agricultural Chemistry. His graduate studies were pursued at Michigan State University in the Department of Biochemistry with the aid of a graduate assistantship and later a National Institute of Health predoctoral fellowship. He received the Master of Science degree in 1963 and will complete the requirements for the degree of Doctor of Philosophy in 1965. He is a member of the American Chemical Society, American Association for the Advancement of Science, and Sigma Xi.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF FIGURES

PART I

THE PRESENCE OF AN INACTIVE ENZYME AND *IN UTERO* TOXICITY IN GALACTOSEMIA

INTRODUCTION

The number of known human enzyme deficiencies has increased at a phenomenal rate within recent years. Although clinical and other studies have been carried out in these disorders, it has been difficult to do molecular studies, because of the inaccessibility of appropriate tissue for laboratory analysis. One of the important questions in patients with these disorders is whether the enzyme protein is completely lacking or altered to an inactive form. The results in individuals with acatalasemia would indicate that the catalase protein is very low and not present as an inactive molecule, but in Glu-6-P dehydrogenase deficiency an inactive form of the enzyme protein may be present.

One of the deficiencies that has been extensively studied is galactosemia. In this disorder Gal-1-P uridyl

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[1]The following abbreviations are used: ADP, adenosine diphosphate; ADPG, adenosine diphosphate glucose; ATP, adenosine triphosphate; CDPG, cytidine diphosphate glucose; DEAE, diethylaminoethyl; EDTA, ethylenediamine tetraacetate; Fru-1-P, fructose-1-phosphate; Gal-1-P, galactose-1-phosphate; Gal-6-P, galactose-6-phosphate; GDPG, guanosine diphosphate glucose; Glu-1-P, glucose-1-phosphate; Glu-6-P, glucose-6-phosphate; Man-1-P, mannose-1-phosphate; TDPG, thymidine diphosphate glucose; TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide, reduced; UDP, uridine diphosphate; UDPG, uridine diphosphate glucose; UMP, uridine monophosphate; UTP, uridine triphosphate; Xyl-1-P, xylose-1-phosphate.

TH

transferase activity is extremely low or completely missing. Therefore, the galactosemics are unable to effectively metabolize galactose, a normal constituent of milk, and symptoms develop at an early age. The heterozygotes for this disorder have a half level of the enzyme and can usually metabolize galactose rapidly enough to avoid complications and symptoms of the disease, but in certain cases the heterozygote may be affected.

The objectives of these experiments were twofold: 1) Purification of the Gal-1-P uridyl transferase, and 2) examination of the galactosemic for the possible presence of an inactive protein. Procedures for the partial purification of the enzyme from calf liver and human erythrocytes were developed. Antibodies were prepared against the purified calf liver enzyme and used to detect cross-reacting material in galactosemia. The cross-reacting material can be interpreted as an indication of the presence of an inactive Gal-1-P uridyl transferase molecule.

During the course of this investigation an opportunity was available to examine some unusual heterozygotes and their families. These heterozygotes had some of the symptoms of galactosemia, suggesting the possibility of <u>in utero</u> toxicity during pregnancy.

LITERATURE REVIEW

Galactose Metabolism

The aldohexose galactose is found throughout nature, but its occurrence in milk is especially significant where it is coupled with glucose as the disaccharide lactose. Since milk is the chief nutrient for young mammals, it would seem necessary to metabolize galactose for energy. Thus in the early 1900's (1) it was shown that galactose could be converted to glycogen. However, it was not until after 1950 that the conversion of galactose to glucose was elucidated.

In 1943 Kosterlitz (2) showed that rabbits fed a high galactose diet accumulated Gal-1-P in their livers. He (3) also showed that a galactose-adapted strain of Saccharomyces cerevisiae could ferment Gal-1-P at a rate equal to that of Glu-1-P. Therefore, he postulated that galactose was metabolized through Gal-1-P which could be converted to Glu-1-P.

A few years later Leloir and his associates established the enzymatic steps and cofactors in this conversion. They (4) were able to show a galactokinase (reaction a) in galactose-adapted Saccharomyces fragilis. They next discovered (5) and identified UDPG (6) as the key coenzyme in the interconversion of galactose and glucose. Leloir (7) later demonstrated in galactose-adapted S. fragilis an

enzyme, UDPGal 4-epimerase (reaction c), that catalyzed the conversion of this coenzyme to a galactose derivative which he identified as UDP Gal. From this observation Leloir postulated two steps in the conversion of Gal-1-P into Glu-1-P: 1) The hyopthetical transfer of UMP from glucose phosphate to galactose phosphate, and 2) the conversion of UDPG to UDPGal by the reaction he had described. Kalckar et al. (8) later demonstrated an enzyme, Gal-1-P uridyl transferase (reaction b), in S. fragilis that catalyzed the reaction postulated by Leloir in step 1.

This pathway for galactose metabolism has become known as the Leloir pathway (9) or the Gal-1-P uridyl transferase pathway (cf. 17) and appears to be the major pathway for galactose metabolism in all organisms. It can be formulated as follows:

a) Galactose + ATP $\rightarrow$ Gal-1-P + ADP

b) UDPG + Gal-1-P $\rightleftharpoons$ UDPGal + Glu-1-P

c) UDPGal $\rightleftharpoons$ UDPG

Sum b and c: Gal-1-P $\rightleftharpoons$ Glu-1-P

In the overall process galactose is converted to Glu-1-P which can be further metabolized by known pathways.

Other minor pathways have been described for the metabolism of galactose and they may play an important role in certain situations. A UDPGal pyrophosphorylase (reaction d) has been described in mammals (10,11), yeast (8), and

plants (12) which with UDPGal 4-epimerase (reaction c) and UDPGlu pyrophosphorylase (reaction e) could convert Gal-1-P to Glu-1-P. This alternate pathway can be formulated as follows:

d) Gal-1-P + UTP $\rightleftharpoons$ UDPGal + PPi

c) UDPGal $\rightleftharpoons$ UDPG

e) UDPG + PPi $\rightleftharpoons$ Glu-1-P + UTP

Sum d, c, and e: Gal-1-P $\rightleftharpoons$ Glu-1-P

The enzyme for reaction d has been shown to increase with age in rat liver (10,11), but even in adult rat liver it is much less active than Gal-1-P uridyl transferase.

It is possible that galactose can be metabolized via a route that doesn't require a uridine coenzyme intermediate. Posternak and Rasselet (13) have shown that phosphoglucomutase can convert Gal-1-P to Gal-6-P and there is some evidence that Gal-6-P can be oxidized by the hexose monophosphate shunt (14). Under normal conditions this oxidative pathway probably has no role in galactose metabolism, but it is of interest that Inouye et al. (14) have identified Gal-6-P in erthrocytes from galactosemic patients who accumulate Gal-1-P in their tissues.

Wells et al. (15,16) have recently identified galactitol in the urine and brain of galactosemic patients and they suggest that the reduction of galactose to galactitol could be an alternate route of galactose metabolism.

Galactosemia

The term galactosemia has come to mean a hereditary condition characterized by a lack of the enzyme Gal-1-P uridyl transferase (reaction b). Several review articles have recently appeared on the subject (17,18,19,20). Affected infants usually appear normal at birth, but after a few days or weeks of milk ingestion vomiting, diarrhea and general listlessness may develop. Other consequences are jaundice, enlargement of the liver and spleen, cataracts, and mental retardation if galactose is continued in the diet. Death is common in the first few weeks of life. These clinical features vary from individual to individual. The common laboratory findings are elevated blood galactose and Gal-1-P, abnormal galactose tolerance, deficiency of Gal-1-P uridyl transferase in red cells, and galactose, amino acids, and protein in the urine.

The first breakthrough in elucidating the biochemical basis of the disease was the discovery by Schwarz *et al.* (21) that the erythrocytes of galactosemics on a milk diet accumulated Gal-1-P and that erythrocytes from galactosemic patients incubated *in vitro* with galactose also accumulated Gal-1-P. This indicated that either the transferase or the epimerase was the block in the pathway of galactose metabolism in galactosemia. Shortly after this finding, Kalckar and co-workers (22,23) were able to show that erythocytes

from galactosemia patients were devoid of Gal-1-P uridyl transferase activity while the other enzymes of galactose metabolism were within the normal range. In galactosemia they (24) subsequently showed that the enzymatic activity was missing in the liver, where most of the galactose is metabolized (24). If normal blood is mixed with galactosemic blood, it retains its ability to metabolize galactose showing that there is not an inhibitory substance in the galactosemic blood.

Although the absence of Gal-1-P uridyl transferase is certainly the cause of galactosemia, it is difficult, at present, to explain all the varied biochemical and clinical features of the disease by the absence of this one enzyme. Holzel et al. (25) suggested that the accumulation of Gal-1-P in the various tissues produces a disturbance of cell metabolism which gives rise to the clinical findings in galactosemia, and Gal-1-P has been shown to inhibit phosphoglucomutase (26,27), Glu-6-P phosphatase (28) and UDPG pyrophosphorylase (29). This concept of Gal-1-P toxicity in galactosemia appears to be well established and is widely accepted. Galactitol has recently been isolated and identified in the urine (15) and brain (16) of galactosemic individuals. This accumulation of galactitol could possibly cause some of the clinical symptoms, especially cataracts, seen in the disorder. Another interesting report on the possible cause of idiocy of galactosemia has been made by Woolley

and Gommi (30). They suggest that the Gal-1-P which accumulates in galactosemia may inhibit the formation of a galactolipid which functions as a serotonin receptor. Therefore, a functional deficiency of serotonin can arise and possibly cause mental defects.

Although the Gal-1-P uridyl transferase pathway is the major means of galactose metabolism and galactosemia results from the absence of one of the enzymes in this pathway, it soon became apparent that some galactosemic patients could metabolize significant amounts of galactose. Eisenberg et al. (31) administered radioactive galactose to a galactosemic patient and concurrently fed menthol to permit the formation of the menthol glucuronide. They estimated that from the 1 gm of galactose administered 75 to 80 percent was not metabolized beyond Gal-1-P which accumulated in the tissues, 3 percent was found in the menthol glucuronide and 20 to 25 percent was not accounted for. The radioactivity in the menthol glucuronide demonstrated that some of the galactose can be utilized by galactosemic patients and that the metabolism probably occurred through uridine mucleotide intermediates.

Segel et al. (32) have observed galactose metabolism in galactosemic patients by injecting intravenously radioactive galactose and measuring the radioactive carbon dioxide in the expired breath. They examined twelve patients who showed the clinical symptoms of congenital galactosemia,

absence of Gal-1-P uridyl transferase in the erythrocytes and inability of hemolysates to oxidize radioactive galactose to carbon dioxide *in vitro*. Three patients could metabolize 1 gm of galactose to a normal extent while the other nine patients were found to metabolize the sugar poorly. There was no evidence that the subgroup who could metabolize galactose differed in symptoms, severity, or clinical histories, and the only correlation in the subgroup was that the three patients were Negro.

Weinberg (33) found, using whole blood or leucocytes *in vitro*, that the blood from one individual was able to convert radioactive galactose to carbon dioxide at a substantially greater rate than observed with other patients in the group. By employing a modification of this *in vitro* procedure, Ng *et al*. (34) have more recently shown that blood from three galactosemics could produce carbon dioxide from radioactive galactose at a higher rate than the other ten patients.

The two most widely discussed views on why the amount of galactose metabolized by galactosemics are the presence of alternate pathways or only partial block in transferase activity. For an alternate pathway the UDPGal pyrophosphorylase has received attention, since this enzyme has been claimed to occur in mammalian liver (11). Several authors have suggested an incomplete block and recently Ng *et al*. (34) have concluded that the enzymatic block in some

galactosemics is incomplete. The basis for this latter conclusion was that some galactosemics could form more UDPHexose than the usual galactosemic when whole blood was incubated with radioactive galactose, but hemolysates could not form labeled UDPHexose from UTP and labeled Gal-1-P which would indicate no UDPGal pyrophosphorylase activity.

A number of investigators have reported a small amount of Gal-1-P uridyl transferase in some galactosemics. Schwarz et al. (35), employing a manometric procedure, have reported some transferase activity in 13 of 23 galactosemic children. Donnell et al. (36), employing a UDPG consumption test, have also found transferase activity in some galactosemics. Hsia and co-workers (37,38), employing various methods for measuring transferase, also reported activity in some galactosemics. They (37) showed that one-third of the galactosemics tested had activity by the oxygen uptake or the UDPG consumption test, but not by both. In a few cases enzymatic activity was found by both methods and in about half the cases neither method showed any enzymatic activity. The discrepancy in the low amount of activity in some galactosemics can also be attributed to variation in the analytical methods.

The frequency of galactosemia has been difficult to assess because many cases die shortly after birth and may not be properly diagnosed or they may not develop symptoms

and go undetected. However, it appears to occur frequently enough that most hospitals with a large children's department will see an appreciable number of cases. After a survey by pediatricians and pathologists in Great Britian it was estimated that 1 in 70,000 children is born with the disorder (35). Hansen et al. (39) have determined the number of heterozygotes in a given population and then by the Hardy-Weinberg equation they calculated the frequency of galactosemia to be 1 in 18,000 births. This frequency is more in the range of other inborn errors which can be determined more accurately such as phenylketonuria (cf. 40). Beutler et al. (41) have also determined the frequency of the carrier state of galactosemia and calculated an expected birth frequency of about 1 in 17,500 births which is similar to that calculated by Hansen et al. (39). Beutler et al. place a different interpretation of their results and state that the calculation is in error in the case of galactosemia. They propose a new genetic abnormality (Duarte variant) for Gal-1-P uridyl transferase deficiency which can not produce true galactosemia and therefore, determined estimates of the birth frequency of galactosemia based on the heterozygote frequency will be too high, unless correction is made for the Duarte variant.

The basis for treatment of galactosemia is to feed a galactose free diet and this diet should be given at the

earliest possible age. There have been several diets described and some have become commercially available (cf. 17,42). The treatment of young infants have had varying degrees of success (cf. 17,42). The amount of galactose ingested and the time of starting of the diet appears to be important. In general, improvement is usually observed but in some cases all the symptoms do not disappear. The treatment of older patients has usually been with an ordinary mixed diet, avoiding only milk, dairy products, and lactose which is sometimes used as filler in vitamin tablets, etc. Since in utero toxicity has been suggested (cf. 17) and a possible case reported (39) it would seem desirable that the expecting heterozygous mother should not consume a large amount of galactose during pregnancy.

Since the diagnosis of galactosemia needs to be made as early as possible so the infant can be placed on a galactose free diet, methods for early detection of the disorder are desirable. A general reducing sugar test can be preformed on the urine in expected cases, and if positive, galactose can be identified by one of several methods. The presence of galactose in the urine is not necessarily diagnostic of galactosemic as other disorders can cause galactose in the urine. Other methods are therefore needed to confirm the disorder. The direct assay for Gal-1-P uridyl transferase in erythrocytes is the best test for diagnosis.

The UDPG consumption test (43), mamnometric assay (44), and radioactive techniques (45,46,47) have been widely employed to measure transferase in erythrocutes. However these procedures require equipment which is not found in every hospital laboratory. Nordin et al. (48) have modified the UDPG consumption test, Schwarz (49) has simplified the manometric assay, and London et al. (50) have developed a simple, inexpensive radioactive test, so they can be used on equipment found in most hospital laboratories and possibly for screening. Recently Beutler et al. (51) have developed a new method for the detection of galactosemia. It is visual and based on a dye-linked system for the estimation of Gal-1-P uridyl transferase. The test appears to be simple, economical, and precise enough to be used for a screening procedure.

Early indications from classical human genetic techniques, such as absence of the disease in parents, high incidence in the offspring of consanquineous marriages and in siblings, and more or less equal occurrence in both sexes, suggested inheritance by a single autosomal recessive gene. However, with the development of techniques for detecting the heterozygote, galactosemia has become one of the best documented examples of a single autosomal recessive gene in human genetics.

Holzer and Komrower (52) used the galactose tolerance test to try to detect the heterozygous carriers of galactosemia and an abnormal tolerance was observed in some

parents but the method did not detect all heterozygous individuals as expected. Later Donnell et al. (53) re-evaluated the galactose tolerance test and identified the heterozygous state in 39 members of galactosemic families. However, the overlap between heterozygous and normal is large and the method must be considered unsuitable for the detection of the heterozygous individual. Hsia et al. (54), employing the original UDPG consumption test of Anderson et al. (43), found a difference between normal and heterozygotes, but considerable overlap was observed.

The first clear-cut demonstration of the detection of the heterozygous carrier of galactosemia was reported by Bretthauer et al. (55). They modified the UDPG consumption test of Anderson et al. (43) to quantitatively assay the Gal-1-P uridyl transferase in erythrocytes and were able to show that parents had transferase levels that were intermediate between the normal and galactosemic. Donnell et al. (36), using the procedure of Bretthauer et al. (55) found a maximum overlap of only 5 percent of the area under the normal distribution curves in a thorough study of the genetics of galactosemia. This was the first clear-cut demonstration of autosomal recessive inheritance in galactosemia.

Concurrently with the study of Bretthauer and coworkers, Kirkman and Bynum (44) developed a manometric method for measuring Gal-1-P uridyl transferase in hemolysates and

achieved a sharp separation between heterozygous carriers of galactosemia and normal. With improved techniques they observed (56) no overlap between heterozygotes and normals. Schwarz et al. (35), using a manometric technique giving maximum activity of Gal-1-P uridyl transferase, found a slight overlap of enzyme levels in the control and heterozygote groups.

Hsia and co-workers (37,38,55,57), using various methods, have sometimes found good discrimination between the heterozygote and normal groups.

From these data it is clear that galactosemia is inherited as a autosomal recessive and that the diseased have none or very little transferase activity and that the heterozygotes have one-half the enzyme activity of normal individuals.

Purification and Properties of Gal-1-P Uridyl Transferase

Although Gal-1-P uridyl transferase is one of the key enzymes in galactose metabolism and its activity is absent in galactosemia, the enzyme has not been extensively purified or studied. Kurahashi and Anderson (58) have purified the enzyme about fifty-fold over the crude extract from calf liver and applied the partially purified enzyme to the determination of Gal-1-P. Kurahashi and Sugimura (59) have purified the enzyme 60-70 fold above the crude extract from

a galactokinase-less strain of Escherichia coli, examined some of its properties and applied the partially purified enzyme to the determination of Gal-1-P. The K_m values for all the substrates were determined and found to be between 1.5 and 4.1 x 10^{-4}. The equilibrium constant was 1.1 in the direction of UDPGal formation. The purified enzyme required cysteine for activity and showed no requirement for Mg, but was inhibited by high Mg concentrations. The pH optimum was between 8.5 and 8.0. Similarly, the pH optimum of the calf liver enzyme has been reported in this range (9).

Molecular Genetics of Human Inborn Errors of Metabolism

The term, inborn errors of metabolism, was coined by Garrod in the early 1900's to explain several disorders that he had observed. Garrod clearly defined biochemical expressions of inherited variability and set forth the idea of inherited blocks in metabolism. His insight was so remarkable for that time that his work was overlooked and it wasn't until the classical work of Beadle and Tatum that the gene-enzyme relationship was well-established. The number of known inborn errors of metabolism has now increased into the hundreds. Their delineation has helped to establish pathways of metabolism, to explain the in vivo role of enzymes, and to understand the physiology and biochemistry of normal individuals.

The question of whether an enzyme is totally absent or is merely in an inactive state in these disorders is of primary concern. The work with microorganisms show that in some cases the mutant enzymes are present but are inactive because of some structural alteration, and even though they are catalytically inactive the proteins will cross react with antibodies prepared against the normal enzyme. In many cases, such as abnormal hemoglobins in humans and tryptophan synthetase in microorganism, the structural alteration has been traced to a single amino acid substitution which would indicate that a change has occurred in the nucleotide code in the DNA and is passed on to the $_{m}$RNA and finally to the protein.

Striking genetic effects on qualitative characteristics of human enzymes have been found in recent years, although the nature of their possible structural differences is difficult to determine because of the inaccessibility of significant quantities of the appropriate tissue. Nevertheless, attempts have been made to determine the absence or presence of mutant enzymes. Robbins (60) prepared antiserum against crystalline rabbit muscle phosphorylase a and b by injecting them into roosters. The antibodies were found to cross-react well with normal human muscle phosphorylase, but no precipitation occurred with extracts from the muscle tissue of a patient lacking phosphorylase. He concluded

that a phosphorylase-like protein in the individual was either completely lacking or that it was modified so extensively that it was both enzymatically and antigenically inactive.

Nishimura et al. (61) produced an antiserum against catalase by injecting a purified preparation of human erythrocyte catalase into rabbits and precipitin reactions were negative with acatalasemic hemolysates. They also concluded that the biochemical defect in acatalasemia appears to be either an absence of erythrocyte catalase or a faulty molecular configuration of it. Ogata and Takahara (62) also could not find any immunological evidence for a mutant protein in hemolysates or acetone extracts of acatalasemic blood by using a quantitative precipitin test. They (63) verified the absence of catalase protein in a spectrophotometric study. Purified catalase from normal individuals showed three absorption bands, at 505, 520, and 625 mμ, but in acatalasemic blood the catalase bands were not detected. Aebi (64) has recently obtained sufficient quantity of catalase from an acatalasemic subject to compare its properties with the enzyme from normal individuals. The catalase from the acatalasemic subject was identical by its mobility on Sephadex G-100 columns, electrophoresis on starch gel, sensitivity towards azide and precipitation by anti-catalase serum. However the concentration of antigenic protein in

the acatalasemic subject differed from that of the normal by a factor of about 200 which is similar to the ratio of catalase activity. It was concluded that true catalase is present in the blood of acatalasemic subjects but only in very small amounts. Thus it is possible that this enzyme defect is not due to a structural gene but rather to a change within the operator gene system.

Kirkman and Crowell (65) have concluded from electrophoresis and titration with TPN that the deficiency of activity of Glu-6-P dehydrogenase in erythrocytes of primaquine sensitive Negroes is due principally to a decrease in the number of molecules of Glu-6-P dehydrogenase. In contrast, Ramot and Bauminger (66) have shown that an inactive Glu-6-P dehydrogenase fraction from erythrocytes of enzyme deficient human subjects could absorb antibody prepared against normal human erythrocyte Glu-6-P dehydrogenase. Since normal enzyme diluted to an activity similar to that of the mutant enzyme was not able to remove the antibody, they suggest that the removal of the antibody was not due to a small amount of active enzyme in the mutant fraction, but due to the presence of an inactive form of Glu-6-P dehydrogenase in enzyme deficient subjects which is immunologically similar to the normal enzyme.

MATERIALS AND METHODS

Chemicals

The biochemicals were purchased as follows: UDPG (isolated from yeast), DEAE cellulose, and Glu-6-P dehydrogenase from the Sigma Chemical Company; TPN from P-L Biochemicals, Incorporated; crystalline phosphoglucomutase and synthetic nucleotide sugars (UDPG, CDPG, GDPG, and ADPG) from CalBiochem. TDPG was synthesized in the laboratory according to the method of Michelson (67,68). Gal-1-P and Xyl-1-P were made in the laboratory according to the method of Hansen et al. (69). Complete Freund adjuvant was obtained from Difco Laboratories.

Calcium phosphate gel was made in large batches by a modification of that given by Colowick (70). One hundred seventy-seven gm of $CaCl_2:2H_2O$ was dissolved in about 6 liters of distilled water. Three hundred and four gm of $Na_3PO_4:12H_2O$ was dissolved in about 2 liters of distilled water and was poured with stirring into the $CaCl_2$ solution. After adjusting the pH of the mixture to pH 7.4 with 6N acetic acid, the gel precipitate was washed with 30-40 liters of distilled water for four times and three times with deionized, distilled water. Concentration of the gel was achieved by

sedimentation by gravity and it was stored in the cold until ready for use.

DEAE cellulose was washed according to the method of Peterson and Sober (71). The fresh or used DEAE cellulose was washed with 1N NaOH until the filtrate was clear. After removing the excess NaOH by washing 5 or 6 times with distilled water, the DEAE cellulose was then converted to the phosphate salt by washing with 0.5N Na_3PO_4 for columns or 0.5N phosphate buffer pH 7.0 for removing hemoglobin from blood. The excess phosphate was removed by washing 5-6 times with distilled water. A thick slurry was kept in the cold until ready for use.

Tissues

Calf liver was obtained from a local slaughterhouse. The calves usually ranged from 1½ to 3 months in age. Human erythrocytes were obtained from the Lansing Regional Blood Center. Both fresh rejected and outdated samples were obtained, but the fresh rejects were higher in enzymatic activity, so these were usually used. Blood samples for the *in utero* toxicity study were obtained from families living in Michigan. Galactosemic blood samples were supplied by Dr. Donnell and Dr. Wells.

Immunological

The purified calf liver Gal-1-P uridyl transferase was emulsified with a syringe with an equal volume of complete Freund adjuvant and injected subcutaneously into Dutch rabbits. A total of three injections were made at weekly intervals. A week after the last injection the rabbit was bleed by making a small nick in the marginal ear vein with a razor blade. Usually, 10 to 20 ml of blood was obtained. The blood was left overnight to clot, centrifuged, and the serum decanted. The gel diffusion test was performed by the method of Ouchterlony (72). The inhibition of the enzyme was performed by a procedure which was similar to that described by Ramot and Bauminger (66).

Assay of Gal-1-P Uridyl Transferase

For the *in utero* toxicity studies the Gal-1-P uridyl transferase in erythrocytes was measured by the method of Bretthauer *et al.* (55). For the other studies the enzyme was measured by methods similar to those described by Kurahashi and Sugimura (59). In a one step assay of transferase Glu-1-P formation is followed spectrophotometrically by TPNH reduction at 340 mμ in a coupled reaction with phosphoglucomutase and glucose-6-P dehydrogenase. The reaction mixture consists of 0.4 μmole of $MgCl_2$; 0.2 μmole of TPN, UDPG, and Gal-1-P; 5 μgm of crystalline phosphoglucomutase;

0.025 units of glucose-6-P dehydrogenase; appropriate amount of Gal-1-P uridyl transferase and 0.1 M glycine buffer pH 8.7 which contained 0.01 M mercaptoethanol to a total volume of 0.50 ml. The assays were performed at 25° C on a Beckman DU spectrophotometer equipped with a Gilford changer and recording attachment (73,74).

A unit is defined as the amount of enzyme that causes the formation of 1 μmole of product per min. under the conditions described above. The formation of 1 μmole of product is based on the molar extinction coefficient of 6.22×10^3 for TPNH at 340 mμ. Specific activity is defined as units per mg of protein. Protein was determined by the method of Lowry et al. (75).

When some of the properties of transferase was studied, a two step assay method was employed so the effects would be on transferase and not on the coupling enzymes. The appropriate amount of transferase was incubated at 25° C with 0.4 μmole of UDPG and Gal-1-P, various sulfhydryl compounds (2μmole) and metals and 0.2 M glycine buffer pH 8.0 to a total volume of 0.2 ml. After eight min. of incubation the reaction was stopped by placing the tubes in a boiling water bath for 1.5 min. The amount of Glu-1-P formed was measured by adding TPN, $MgCl_2$, phosphoglucomutase and Glu-6-P dehydrogenase and observing the total change in optical density at 340 mμ.

In crude preparations 6-P gluconic acid dehydrogenase was present and interfered with the calculation of the transferase activity. 6-P gluconic acid dehydrogenase was therefore measured according to the principle of Horecker and Smyrniotis (76) with 0.2 μmole of TPN and 6-P gluconic acid, 0.4 μmole $MgCl_2$ and 0.1 M glycine buffer pH 8.7 in a total volume of 0.50 ml. This procedure was employed for most of the one step assays for Gal-1-P uridyl transferase and taken into account in calculating the activity. Also in crude preparation a pyrophosphatase was present which hydrolyzed UDPG to Glu-1-P. Therefore, a control without Gal-1-P was necessary to measure this activity.

RESULTS

I. Purification of Gal-1-P Uridyl Transferase from Calf Liver

The livers from calves were obtained as soon as possible after death, sliced into small pieces and packed in dry ice. They were transported to the laboratory and kept frozen until ready for use. Purification of the enzyme was conducted in the following manner with all steps being carried out at 0-4° C and with distilled, deionized water. Centrifugation, unless otherwise stated, was for 20 min at 23,500 x g.

Step 1. One kilogram of frozen calf liver was diced and extracted with 2000 ml of 0.03 M KOH-0.005 M EDTA Na_4 in a large Waring blender for 2 min. The extract, which was originally pH 8.6, was adjusted to pH 6.8 with 6.0 N acetic acid. Four hundred ml of 2% protamine sulfate was added and after stirring for 15 min, the mixture was centrifuged.

Step 2. To the supernatant from step 1, solid ammonium sulfate was added slowly with stirring until 50% saturation (0.298 gm/ml). After stirring 30 min the solution was centrifuged. The supernatant was discarded and the precipitate dissolved in a minimum of cold water and dialyzed for 3 hrs against 2 changes of about 10 liters of water.

Step 3. To the dialysate of step 2, calcium phosphate gel was added until the gel to protein ratio was 0.8. After stirring for 10 min the solution was centrifuged. To the supernatant four times the original amount of calcium phosphate gel was added. After stirring for 2 hrs the solution was centrifuged and the supernatant discarded. The precipitated gel was suspended in 1500 ml of 0.02 M phosphate buffer pH 8.0 and after eluting overnight the mixture was centrifuged.

Step 4. The supernatant from step 3 was placed directly on a DEAE cellulose column (30 x 2 cm). The activity was eluted with a gradient of phosphate buffer pH 7.0. A gradient system similar to that described by Hurlbert et al. (77) was employed. Starting with water in a 500 ml mixing flask and 0.05 M buffer in the reservoir, the elution was continued until the enzyme started to come through. The elution of the enzyme was completed with 0.08 M buffer in the reservoir.

Step 5. Tubes from step 4 containing the enzyme were combined and diluted with 1.5 volumes of cold water. The enzyme was adsorbed on a small column (7 x 1 cm) of DEAE cellulose, then eluted with 0.16 M phosphate buffer at pH 7.3.

Step 6. Tubes from step 5 containing the enzyme were combined and solid ammonium sulfate added until 30% saturation (0.168 gm/ml). After stirring for 15 min the solution was centrifuged. Ammonium sulfate was again added to the supernatant until 50% saturation (0.119 gm/ml). After stirring for 15 min the solution was centrifuged. The supernatant was discarded and the precipitate dissolved in a small amount of 0.05 M phosphate pH 7.0 which contained 0.01 M mercaptoethanol.

A summary of the procedure is given in Table 1. An overall purification of about 500 fold was achieved with a yield of about 23 percent. The purification is probably higher because the protamine sulfate usually resulted in precipitation of some protein. The procedure has been repeated several times with similar results. In step 3 the protein concentration should be about 50 mg/ml and the gel concentration should be about 25 to 30 mg/ml for best results. After the DEAE cellulose column the activity should be concentrated as soon as possible because the enzyme is very unstable in the dilute solution.

The elution pattern from the DEAE cellulose column is given in Figure 1. A large amount of protein was not absorbed while the sample was being placed on the column. The peak of protein was eluted with 0.05 M phosphate buffer, while the activity was eluted in a sharp peak with 0.08 M phosphate buffer. Tubes number 206 through 246 were combined for the next step.

Table I. Purification of Gal-1-P Uridyl Transferase from Calf Liver

Step	Ml	Units/ml	Total Units	Protein	S.A.
Protamin sulfate	2340	0.852	2000	34.2	.025
Ammonium sulfate (0-50%)	785	2.38	1870	53.4	.044
$Ca_3(PO_4)_2$ gel	1595	0.700	1120	0.57	1.23
DEAE cellulose (column)	635	1.23	780	0.14	8.79
DEAE cellulose (concentration)	35	15.1	530	1.4	10.8
Ammonium sulfate II (30-50%)	2.2	217.0	480	17.7	12.4

Assay conditions, unit of enzyme, and specific activity are described in text. Protein is given as mg/ml.

Figure 1. Elution pattern from DEAE cellulose.

The column and method of elution are described in the text. Solid line represents protein as determined at 280 mμ, and the dash line represents activity of Gal-1-P uridyl transferase.

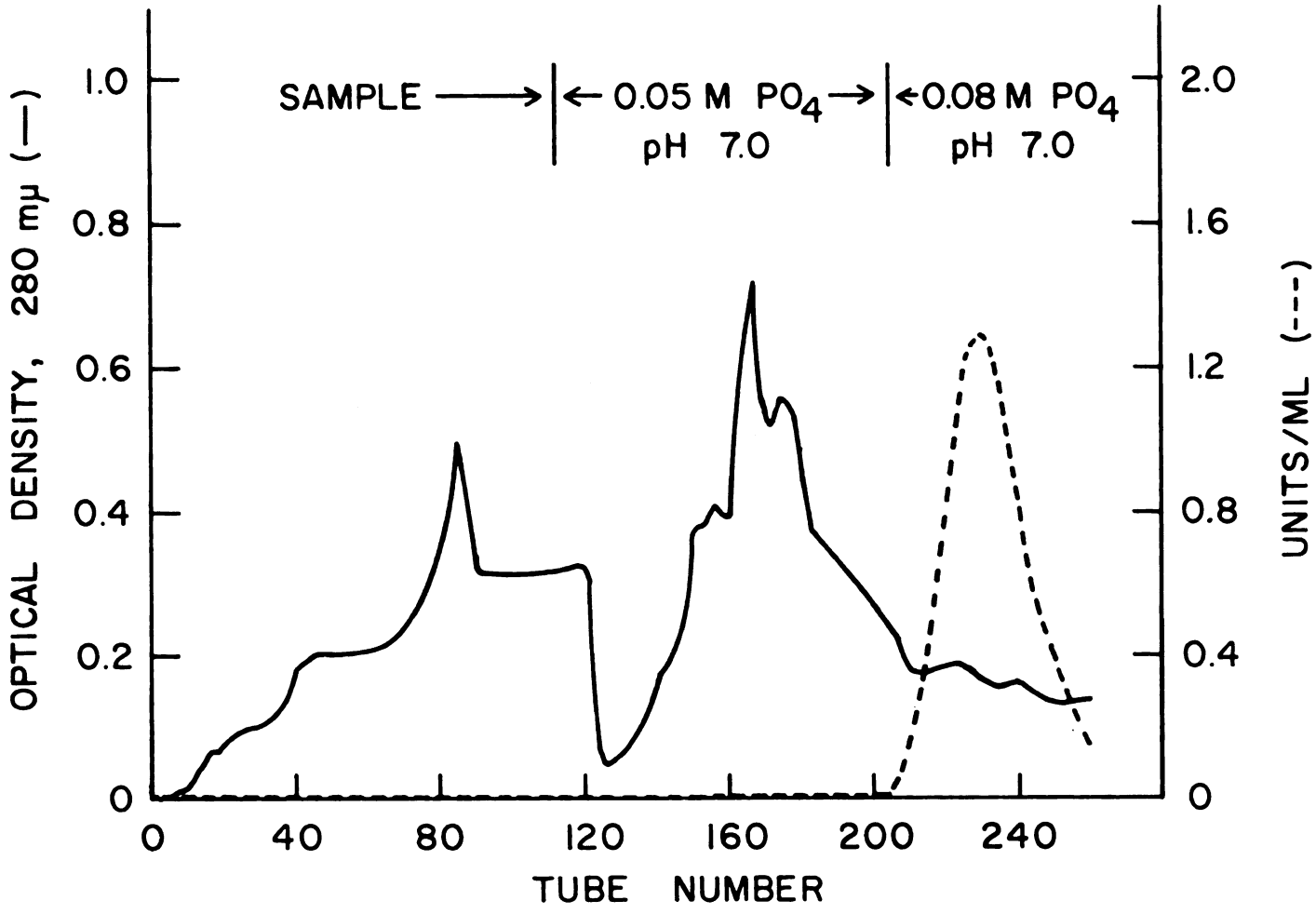

SAMPLE
← 0.05 M PO4 →
pH 7.0
←0.08 M PO4
pH 7.0
OPTICAL DENSITY, 280 mμ (—)
UNITS/ML (---)
1.0
0.8
0.6
0.4
0.2
0
2.0
1.6
1.2
0.8
0.4
0
0
40
80
120
160
200
240
TUBE NUMBER

Figure 1

Purity

On cellulose acetate electrophoresis the enzyme from the last step of purification gave one major protein band. If the cellulose acetate strip was cut in half and one-half stained for protein and the other half cut into small pieces, eluted and assayed for transferase, the peak of activity corresponded to the protein band. At this stage the enzyme was thought to be fairly homogeneous. However, when antibodies were prepared against the enzyme, multiple precipitin lines were observed on gel diffusion and in the analytical ultracentrifuge the pattern became dispersed as the protein moved down the cell. When the protein was electrophoresized on the more resolvable polyacrylamide gel, several major and minor bands were observed. When one of the gel columns was eluted and assayed for transferase, the activity corresponded to one of the major bands. The purity can be estimated roughly at 10 to 30%.

Stability

For pH stability 100 gm of calf liver was extracted in the usual manner and purified through step 2. One ml of the enzyme was mixed with 1 ml of each of pH 6.0, 7.0 and 8.0 phosphate buffer 0.1 M. The mixtures were incubated at 25° C and assayed at the various times which are given in Table II. The enzyme was stable at pH 7.0 and 8.0, but it was unstable

Table II. pH Stability of Gal-1-P Uridyl Transferase

Time	Activity units/ml		
	pH 6.0	pH 7.0	pH 8.0
0	1.06	1.00	1.03
1 hr	.96	1.00	1.00
13 hr	.71	1.06	1.03
18 hr	.60	1.03	.93

at pH 6.0 where it lost almost half of its activity in 18 hours. This trend in pH stability was also observed in early purification procedures since if the calcium phosphate gel or DEAE cellulose steps were carried out at acid pHs, a loss in enzyme activity was observed.

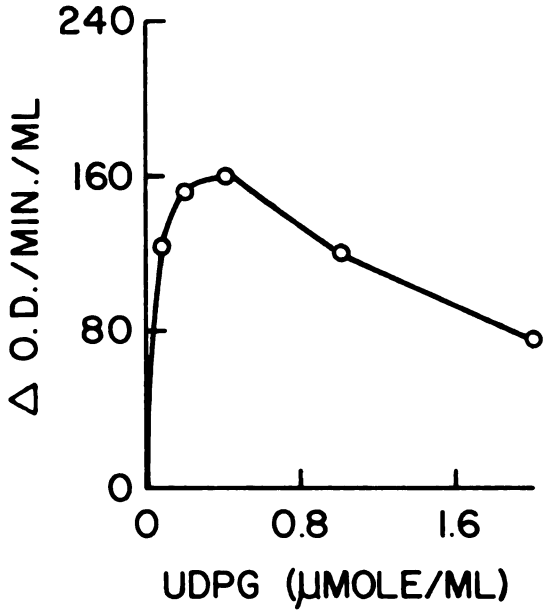
Sulfhydryl reagents also appeared to stabilize the enzyme.

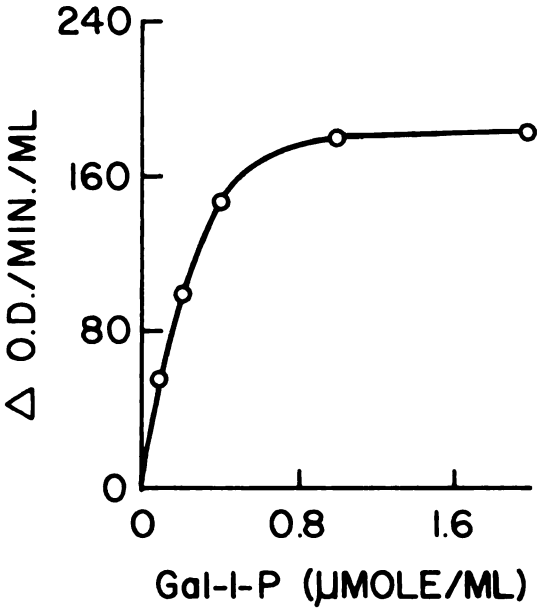
Effect of Enzyme and Substrate Concentration

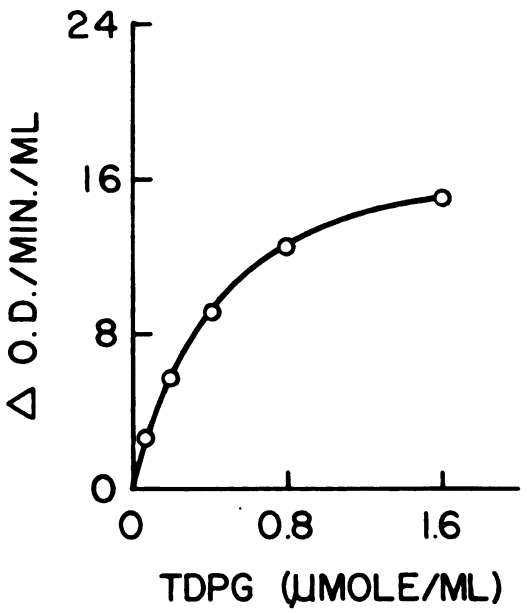
In the one step procedure the response was linear with respect to enzyme concentration until a change in optical density of about 0.02 per min. A range of 0.008 to 0.02 change in optical density per min was usually employed in assaying the enzyme. Since in the two step assay the reaction was linear up to 12 min, eight min was employed for routine determinations.

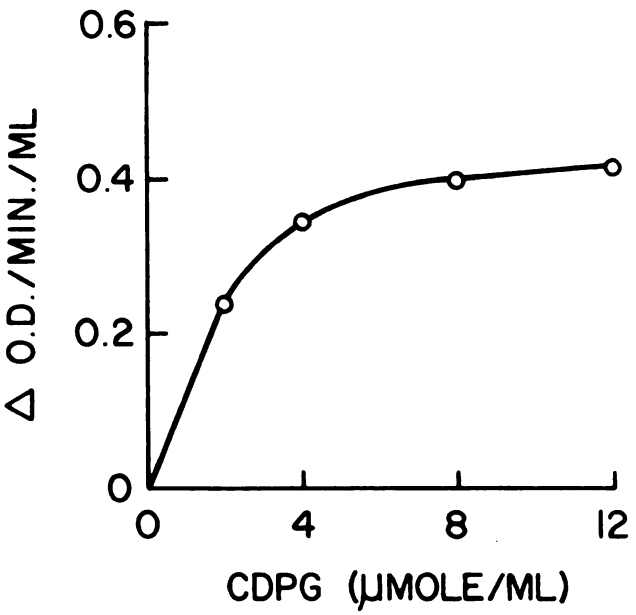
The effects of concentration of various substrates on the reaction rate are given in Figure 2. The optimum concentration for UDPG is 0.4 μmole/ml and at higher concentrations of UDPG the reaction was inhibited. Therefore, a concentration of 0.4 μmole/ml was used to measure the enzyme. The optimum concentration for Gal-1-P was between 0.4 and 1.0 μmole/ml. 0.4 μmole/ml of Gal-1-P was usually employed in the reaction mixtures. The optimum concentrations for TDPG and CDPG were much higher.

Figure 2. Effect of substrate concentration on the reaction rate.


240
160
80
0
Δ O.D./MIN./ML
0
0.8
1.6
UDPG (μMOLE/ML)


240
160
80
0
Δ O.D./MIN./ML
0
0.8
1.6
Gal-1-P (μMOLE/ML)


24
16
8
0
Δ O.D./MIN./ML
0
0.8
1.6
TDPG (μMOLE/ML)


0.6
0.4
0.2
0
Δ O.D./MIN./ML
0
4
8
12
CDPG (μMOLE/ML)

Substrate Specificity

The substrate specificity of purified Gal-1-P uridyl transferase is given in Table III. The enzyme was somewhat active in catalyzing the hexose transfer reaction with the pyrimidine molecules, but there was no detectable activity with the purine diphosphate glucoses or other sugar phosphates. After the natural substrates, Gal-1-P, Glu-1-P, UDPG, and UDPGal, TDPG was the most active. This probably is a non-specific catalysis of the enzyme since the UDPG/TDPG activity ratio remained constant throughout purification. Also, the activity was not a small contamination of UDPG in the TDPG because in an end point assay with high enzyme concentration the TDPG showed 95.5% of the expected value. The catalysis with CDPG as substrate is less certain. At a concentration of 0.4 μmole/ml (Table III), the reaction is barely detectable, but at higher concentrations of substrate the reaction rate is increased to about 0.25% that of UDPG and appears to be non-specific.

Effect of Sulfhydryl Reagents

The effect of sulfhydryl reagents on the catalysis rate is given in Table IV. All three of the compounds tested stimulated the enzyme to about the same extent, although mercaptoethanol always appeared to be slightly more active than glutathione or cysteine. Therefore, mercaptoethanol was regularly used to stabilize the enzyme.

Table III. Substrate Specificity of Gal-1-P Uridyl Transferase

Substrate	Percent of Activity
1. Nucleotide sugars	
UDPG	100
TDPG	8.5
CDPG	.07
ADPG	< .01
GDPG	< .01
2. Phosphate sugars	
Gal-1-P	100
Man-1-P	< .01
Fru-1-P	< .01
Xyl-1-P	< .01
Gal-6-P	< .01

Table IV. Effect of Sulfhydryl Reagents on Gal-1-P Uridyl Transferase

Reagent	Change in O. D. Expt. 1	Expt. 2
None	.084	.072
Mercaptoethanol	.160	.112
Glutathione	.142	.100
Cysteine	.138	.104

The assay is the same as described in the text for the two step assay, except no metal ions were used.

Effect of Metal Ions

The effect of magnesium on the catalytic activity of the transferase in the one step assay is shown in Figure 3. Magnesium was stimulatory at low concentration but was inhibitory at high concentration. In order to check if this effect was directly on transferase or the coupling enzymes, a two step procedure was performed and the results are shown in Figure 4. Under these conditions only the inhibition was observed. Since phosphoglucomutase is known to require a small amount of magnesium, the stimulation was probably due to this reaction. Several other divalent metal ions were tested and all were inhibitory with copper being the most affective.

II. Purification of Gal-1-P Uridyl Transferase from Human Erythrocytes

The erythrocytes from four pints of blood were washed twice with physiological saline and held frozen overnight to rupture the cells. The frozen cells were thawed and diluted with an equal volume of water to form the hemolysate.

Step 1. The purpose of this step was to remove the hemoglobin. The procedure adopted was modified from that described by Hennessey et al. (78). An equal volume of a thick slurry of DEAE cellulose was added to the hemolysate, and after stirring for 1 hour, the mixture was poured into

Figure 3. Effect of magnesium on the reaction rate in the one step assay.

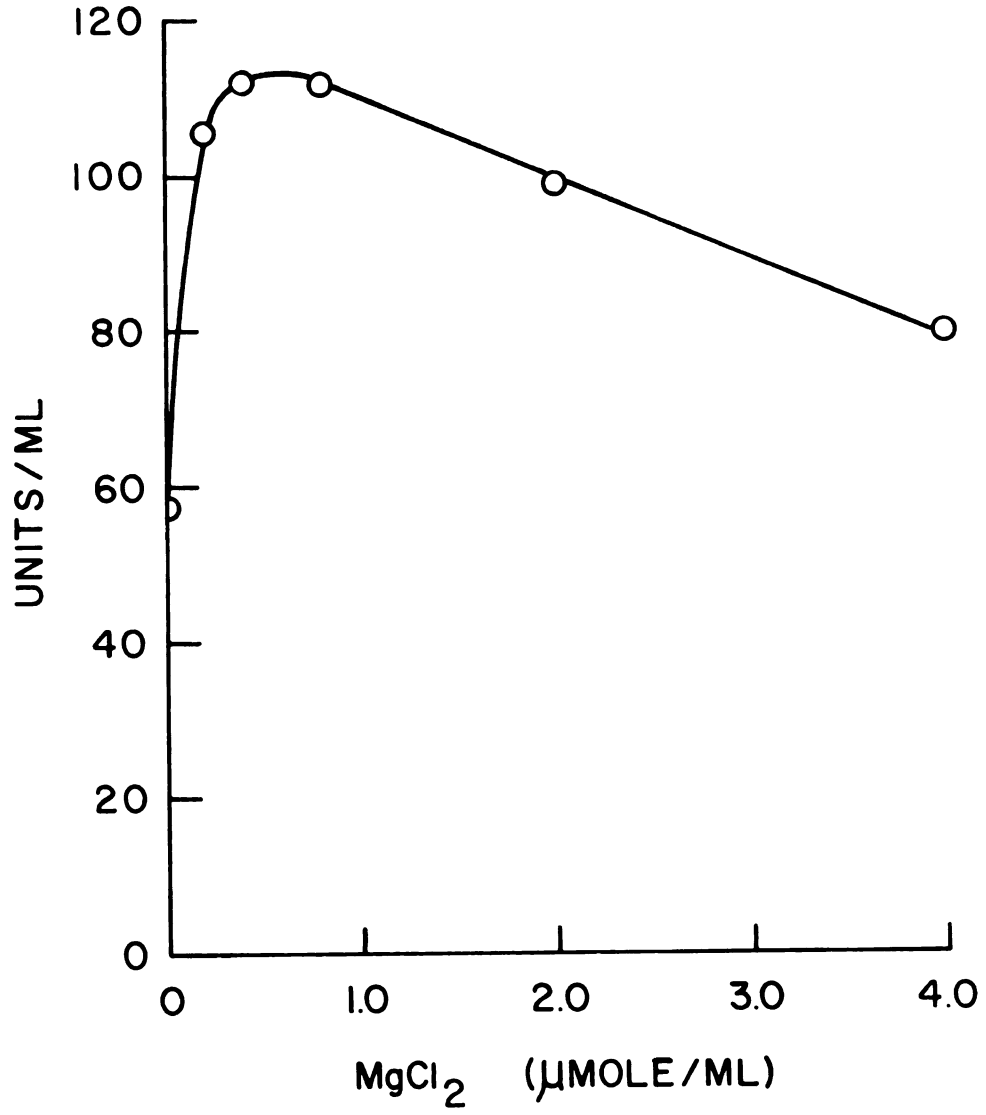
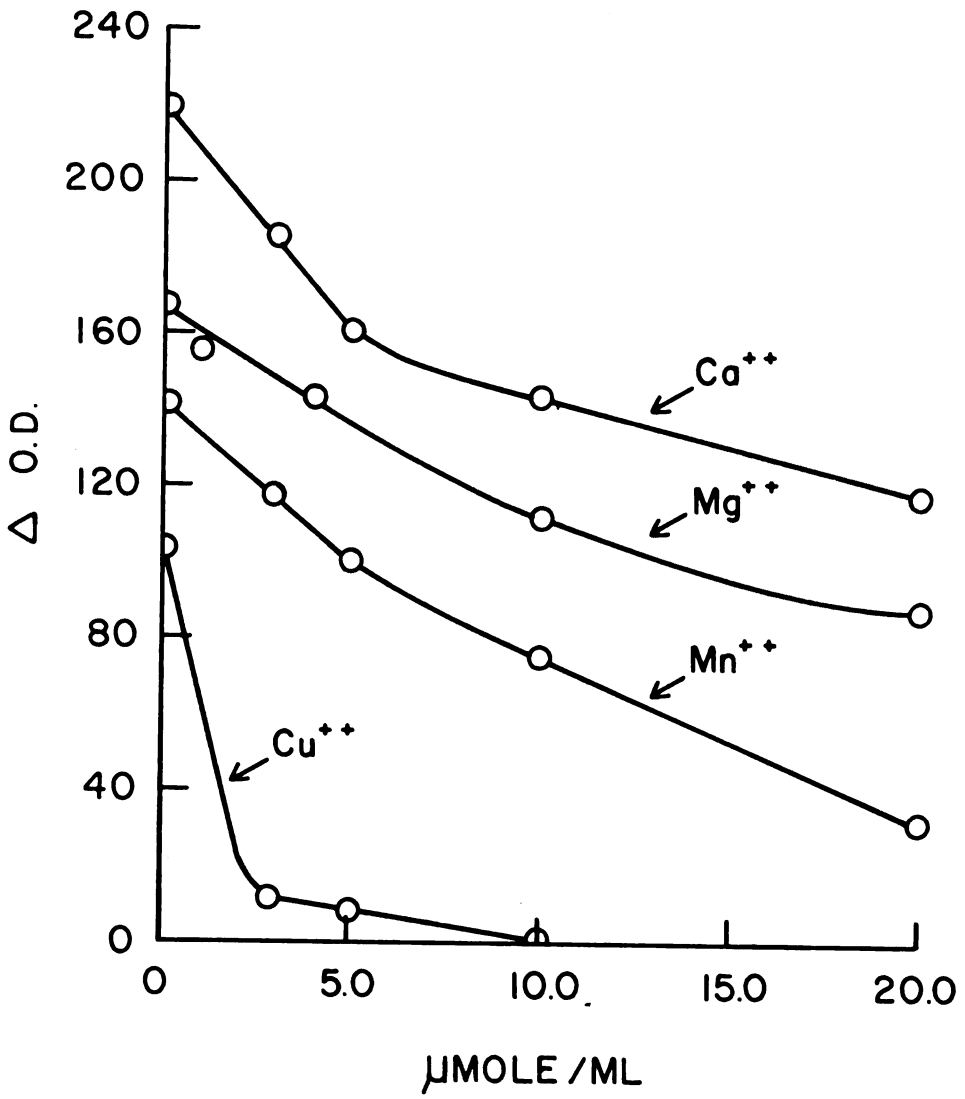

120
100
80
60
40
20
0
UNITS/ML
0
1.0
2.0
3.0
4.0
MgCl2 (μMOLE/ML)

Figure 4. Effect of metal ions on the reaction rate in the two step assay.


240
200
160
120
80
40
0
Δ O.D.
Ca++
Mg++
Mn++
Cu++
0
5.0
10.0
15.0
20.0
μMOLE / ML

a large fritted glass funnel. The DEAE cellulose on the funnel was washed with 0.0025 M phosphate buffer pH 7.0 until the red color was removed. The DEAE cellulose was then removed from the funnel and the enzyme eluted with 0.5 M KCl for one hour and filtered from the DEAE cellulose.

Step 2. To the filtrate from step 1, solid ammonium sulfate was added until 50% saturation (0.298 gm/ml). After stirring for 20 min the solution was centrifuged. The supernatant was discarded and the precipitate dissolved in water. The solution was dialyzed for 3 hours against two changes of water of about 10 liters each.

Step 3. To the dialysate of step 2, calcium phosphate gel was added to reach a gel to protein ratio of 1.2. After stirring for 15 min the solution was centrifuged. Eight times the original amount of calcium phosphate gel was added to the supernatant. After stirring for 2 hours the solution was again centrifuged. The precipitated gel was eluted with 0.05 M phosphate buffer pH 8.0 for 2 hours and centrifuged to remove the gel.

Step 4. To concentrate the supernatant of step 3, solid ammonium sulfate was added until 60% saturation and after stirring for 15 min the solution was centrifuged. The supernatant was discarded and the precipitate dissolved in a small amount of water.

A summary of this purification procedure is given in Table V. An overall purification of about 500 fold with a yield of 24% was achieved. Several additional steps, such as DEAE cellulose column and AlCγ gel, were attempted without success. The main problem was that the enzyme became too dilute and lost activity before concentration. The above procedure gave a transferase that was free of enzymes, such as 6-P gluconate dehydrogenase and pyrophosphatase, that interfere with the measurement of the enzyme. Thus this preparation procedure can be used to study the enzyme from human erythrocytes.

III. Immunological Studies

Precipitation of Enzymes from Calf Liver and Human Erythrocytes by Antiserum

In order to measure the amount of antibody-antigen interaction, the antigen and antibody were mixed with Tris-HCl buffer pH 7.8 at a concentration of 0.1 M, incubated for 30 min at 25° C, and centrifuged. From the amount of enzymatic activity in the supernatant, the amount of precipitation or percent inhibition could be calculated. A duplicate tube of control antibody was always run.

The percent inhibition of the calf liver enzyme at various concentrations of serum is shown in Figure 5. The inhibition at low levels was proportional to serum concentrations, but became nonlinear at high serum concentrations.

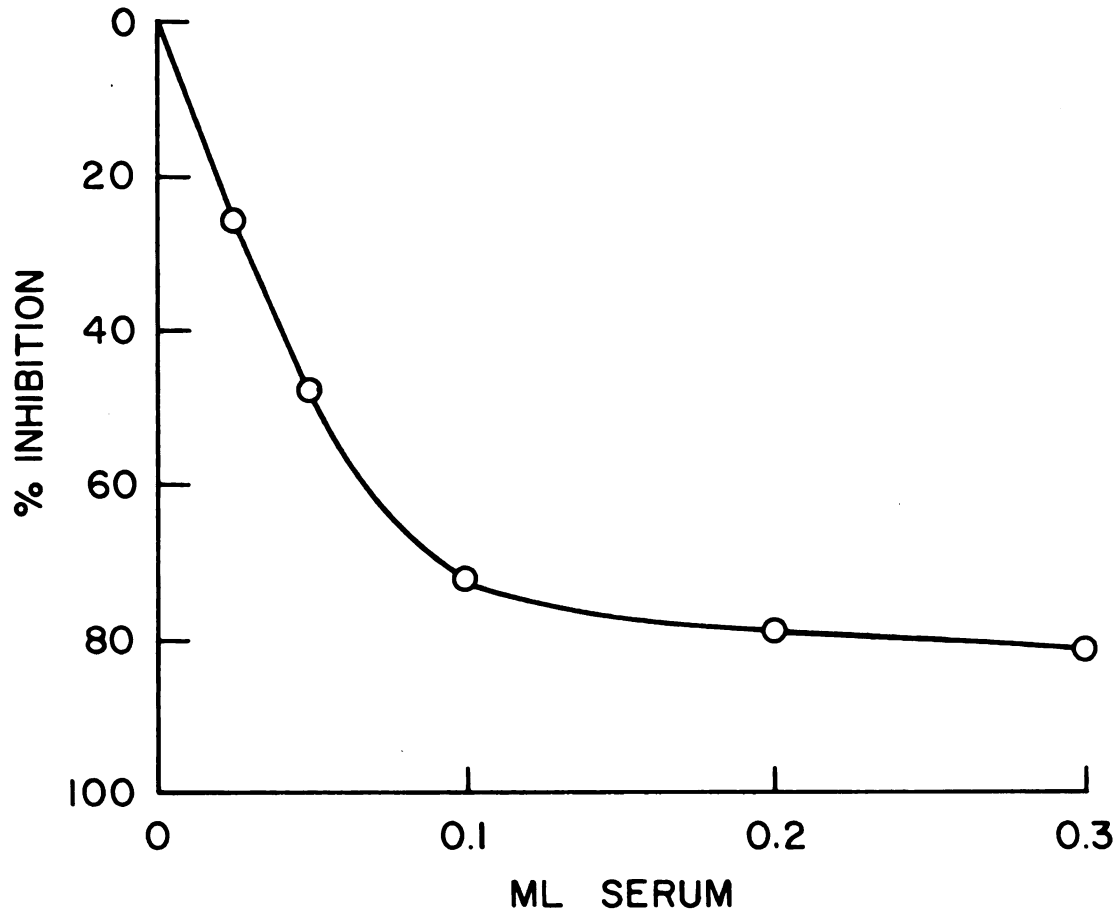
Table V. Purification of Gal-1-P Uridyl Transferase from Human Erythrocytes

	Ml	U/ml	Total Units	Protein	S.A.
Hemolysate	2020	.0177	35.9	160	.00011
DEAE cellulose	2145	.0105	22.4	5.3	.0020
Ammonium sulfate (0-50%)	268	.071	19.0	17.7	.0040
$Ca_3(PO_4)_2$ gel	340	.035	11.9	.66	.053
Ammonium sulfate (0-60%)	9.5	.90	8.5	15.6	.058

Assay conditions, units (U), and specific activity (S. A.) are described in text. Protein is given as mg/ml.

Figure 5. Precipitation of calf liver enzyme with anti-serum.

0.01 ml of a 1-10 dilution of enzyme (0.014 units) was incubated at 25° C for 30 min with 0.025 ml of Tris-HCl buffer 1.0 M, pH 7.8 and water and/or serum to a total volume of 0.335 ml. The mixture was centrifuged and an aliquot (0.025 ml) of the supernatant assay for activity. The difference between the activity in control serum and antibody serum was used to calculate the percent inhibition.


0
20
40
60
80
100
% INHIBITION
0
0.1
0.2
0.3
ML SERUM

This is probably due to a small amount of Gal-1-P uridyl transferase in the antiserum which would not be precipitated and could become meaningful at high concentration of serum.

The inhibition of the human erythrocyte enzyme by the antibody to the calf liver enzyme is given in Table VI. The human erythrocyte enzyme was not as effective as the calf liver enzyme in precipitating the antibody, but did as expected show some inhibition or cross reaction. 0.05 ml of antiserum would precipitate about 0.007 units of calf liver enzyme but only 0.0005 units of human erythrocytes enzyme.

Precipitation of the Antibody by Blood Extracts from Galactosemics

Blood from galactosemic patients was fractionated through the second step in the purification procedure for erythrocytes. This fraction (0.06 ml) was then incubated with antigenic and control serum (0.06 ml) and buffer for 30 min at 25° C and centrifuged. An aliquot (0.1 ml) was incubated with calf liver enzyme for 30 min at 25° C as described in Figure 1, and the activity in the supernatant was measured. The results are summarized in Table VII. The fraction from galactosemic blood appeared to precipitate rabbit antibody prepared in response to the calf liver transferase. Thus it is probable that a protein is present in the blood of the galactosemic which is immunologically similar to the normal transferase but catalytically inactive.

Table VI. Precipitation of Human Erythrocyte Enzyme with Rabbit Antiserum

	Δ O.D./min	% I
Experiment 1		
Control serum	.0116	20
Antiserum	.0088	
Experiment 2A		
Control serum	.0172	16
Antiserum	.0144	
Experiment 2B		
Control serum	.0188	28
Antiserum	.0136	

In experiment 1, 0.1 ml of the enzyme (0.0048 units) was incubated for 30 min at 25° C with 0.1 ml of serum and 0.02 ml 1.0 M Tris-HCl buffer pH 7.8. The mixture was centrifuged and the supernatant assayed for activity. The difference between the activity in the control serum and antibody was used to calculate the percent inhibition. Experiment 2 was similar to experiment 1, except 0.05 ml of antibody and 0.05 ml of antigen in 2A and 0.10 ml of antigen in 2B were used.

Table VII. Precipitation of Antibody by a Fraction of Galactosemic Blood

	Percent Inhibition of Calf Liver Enzyme
Control 1	41
Patient 1	21
Control 2	47
Patient 2	26

In the control, 0.06 ml of water was added to each of two tubes. 0.06 ml normal rabbit serum was added to one tube, and 0.06 ml of immune rabbit serum was added to the other tube. To two other tubes, 0.06 ml of a fraction of galactosemic blood was added. 0.06 ml of normal rabbit serum was added to one tube, and 0.06 ml of immune rabbit serum to the other tube. After incubating for 30 min, the mixtures were centrifuged; and an aliquot (0.1 ml) of each was incubated with calf liver enzyme for 30 min. After centrifuging, the supernatants were assayed for activity. The difference between the activity of samples incubated with normal rabbit serum and immune rabbit serum was used to calculate the percent inhibition.

IV. Symptoms of Galactosemia in the Heterozygote and Possibility of in utero Toxicity in These Individuals

In the study of the frequency of galactosemia (40), a patient was observed in a hospital for the mentally retarded which had some of the symptoms of galactosemic, for example, eye cataracts and mental retardation. Upon measuring the Gal-1-P uridyl transferase in the erythrocytes, the level was found to be within the range of heterozygous individuals. It was possible in this case to do a family study which revealed that the mother and several siblings had transferase levels within the heterozygous range while the father and other siblings had levels within the normal range.

In a second case, the child was thought by pediatricians to be a galactosemic since jaundice was present and reducing substances were found in the urine. The jaundice disappeared upon the administration of a galactose-free diet. However, upon measuring the Gal-1-P uridyl transferase in the erythrocytes, the value was within the range of heterozygous individuals. In a family study it was again the mother who had transferase levels in the heterozygous range. The infant was also thought to have biliary atresia which complicates the symptoms in this case, but since biliary atresia has been described (cf. 17) in galactosemia it is possible that this complication resulted from galactose toxicity.

A summary of the transferase levels in the two families is given in Table VIII. Since transferase levels below 4.0 have been shown to be indicative of heterozygous individuals (55), the patient and mother in both families were within this range while the fathers were clearly within the normal. Thus it is possible that these two children are unusual heterozygotes that show some of the symptoms of galactosemia. The symptoms in the children may have resulted from inadequate metabolism of galactose by the mother during maternity.

Table VIII. Gal-1-P Uridyl Transferase Levels in Erythrocytes of Families with Possible *in utero* Toxicity

	μm of UDPG consumed per ml erythrocyte per hr
Family 1	
Patient	2.4
Mother	2.4
Father	5.3
Siblings	6.8, 6.5, 2.4, 2.9
Family 2	
Patient	3.8
Mother	2.0
Parents of Mother	
Mother	3.4
Father	3.4
Father	6.4

DISCUSSION

It is well-established that galactosemia is an inborn error of metabolism in which the enzyme Gal-1-P uridyl transferase is very low or completely missing in the tissues. It is of interest to know if the enzyme protein is only modified to an enzymatically inactive form or is completely lacking. If the mutation is in the structural gene an altered inactive enzyme would be expected, but if the mutation is in the regulator gene a decrease or complete loss of activity would occur. Of the human enzyme deficiencies studied, both types of mutations appear to exist. In phosphorylase deficiency (60) and acatalasemia (61,62,63,64) the enzyme proteins appear to be either completely lacking or very low and in acatalasemia (64) the mutation has been suggested to occur within the operator gene system. However, in Glu-6-P dehydrogenase deficiency, an inactive Glu-6-P dehydrogenase fraction appears to exist (66).

In consideration of an inactive Gal-1-P uridyl transferase the approach was to purify the enzyme, prepare antibodies against it, and then see if the antibody would cross-react with a fraction from erythrocytes from galactosemic patients.

After preliminary purification of transferase from galactose-adapted yeast, calf liver and human erythrocytes, calf liver was finally chosen as the tissue for purification of the enzyme, because it was readily available, had high levels of activity, and was expected to cross react with human Gal-1-P uridyl transferase.

The enzyme from calf liver was purified 500 fold from the crude extract with a 23% yield (Table 1). The purified enzyme was fairly specific (Table III). For the nucleotide moiety the enzyme only reacted to a slight extent with TDPG and very slightly with CDPG in addition to UDPG as the major substrate. For the sugar moiety the enzyme appeared to be specific for glucose and galactose. The calf liver enzyme was stimulated 30 to 50% with sulfhydryl reagents. This stimulation is not as much as that reported for the E. coli enzyme which had an absolute requirement for sulfhydryl reagents (cysteine) for activity (59). The purified enzyme had no requirement for divalent metals but all the metals tested were inhibitory. This phenomena has been reported for magnesium for the E. coli enzyme (59). The purified enzyme was inhibited with high concentrations of UDPG.

Antibody was prepared against the purified Gal-1-P uridyl transferase by injecting the enzyme into rabbits. Since the enzyme showed several lines of precipitation on gel diffusion with the antibody and several lines on disc electrophoresis, an inhibition method was used to determine

the amount of antibody-antigen interaction. The calf liver enzyme (Figure 5) and normal human erythrocyte enzyme (Table VI) were both precipitated with the antibody. However, the human erythrocyte enzyme did not react as effectively as the calf liver enzyme. A fraction from the erythrocytes of patients with galactosemia purified through step 2 of the fractionation procedure for erythrocytes (Table V), precipitated the antibody (Table VII). It is assumed that the substance in the erythrocytes responsible for the precipitation of the antibody is inactive protein. A small amount of active Gal-1-P uridyl transferase could give this effect but no activity of Gal-1-P uridyl transferase could be detected in the fraction and by using the purification procedure to concentrate the enzyme, less than 5% of the normal amount should be detectable. Therefore, the substance in the erythrocytes of the galactosemic that precipitates the antibody appears to be catalytically inactive Gal-1-P uridyl transferase.

Two possible cases of <u>in utero</u> toxicity of galactose have been observed. The first case was found in a school for the mentally retarded. The child, his mother and some siblings had levels of Gal-1-P uridyl transferase in their erythrocytes which indicate that they were heterozygous while the father and other siblings had values which indicate that they were normal for the galactosemia trait.

It is of interest why the one child, which was heterozygous, had symptoms of galactosemia and was severely affected while the other heterozygous siblings were not affected. This could have possibly been due to the diet, especially milk and dairy products, of the mother during pregnancy or possibly the other siblings did show some of the symptoms at one time or another, but they weren't as severe as the mentally retarded child. In the second case the mother and child also had levels of transferase in their erythrocytes indicative of heterozygous individuals.

In both cases it was the mother with transferase levels which were low and in the heterozygous range as was the child's. Since women are usually encouraged to drink milk during pregnancy, it is possible that in mothers heterozygous for galactosemia the galactose is increased significantly in the blood and a toxic level of Gal-1-P or some other product may reach the fetus.

Other authors have suggested _in utero_ toxicity. Since improvement of galactosemia patients on a galactose-free diet is sometimes limited, even when the diet was started fairly early in infancy, Clay and Potter (79) suggested that this may be due to damage occurring _in utero_. Schwarz _et al_. (80) found an increased level of Gal-1-P in the blood of one galactosemic infant who had been given no milk suggesting _in utero_ toxicity. The fact that the

galactose tolerance curve tends to be abnormal in late pregnancy (43), lower birth weight for galactosemics than for their normal sibs (37), and induction of cataracts in fetal rats by feeding the pregnant mothers a high galactose diet (81), are also suggestive of *in utero* toxicity.

SUMMARY

Gal-1-P uridyl transferase has been purified about 500 fold from extracts from calf liver and some of its properties have been studied. The enzyme is fairly specific for substrates reacting slightly with TDPG and very slightly with CDPG. The enzyme was inhibited with divalent metals and high concentration of UDPG and stimulated with sulfhydryl compounds.

Antibodies were prepared against the purified protein and the antibody precipitated both the calf liver and human erythrocyte Gal-1-P uridyl transferase. A fraction from galactosemia blood would also precipitate the antibody suggesting the presence of an inactive Gal-1-P uridyl transferase protein in the galactosemic.

Two possible cases of *in utero* toxicity in individuals heterozygous for galactosemia were found. In both cases the mother and the affected child had transferase levels in the low heterozygous range and the child showed some of the symptoms of the galactosemic suggesting *in utero* toxicity.

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PART II

URINARY MUCOPOLYSACCHARIDES IN PATIENTS WITH HURLER'S SYNDROME, THEIR FAMILIES AND NORMAL MAN

INTRODUCTION

Several hereditary disorders have been reported in which there are abnormal metabolism and excretion of mucopolysaccharides. Of these, Hurler's syndrome is the only severe mucopolysaccharide disorder that has been conclusively documented. It is well-established that chondroitin sulfate B and/or heparitin sulfate are excreted in the urine and accumulate in the tissues in abnormal amounts in these individuals. Hurler's syndrome is not a single entity as several different types appear to exist. From genetic studies, it is clear that two hereditary forms, an autosomal recessive and a sex-linked recessive, exist. It now becomes clear, from mucopolysaccharide excretion patterns, that four or five different types may exist.

The heterozygotes of Hurler's syndrome are asymptomatic and the reports concerned with the detection of heterozygotes by abnormal urinary mucopolysaccharides are not consistent.

The excretion of mucopolysaccharides in the urine of normal man appears to be more complicated than originally observed. Chondronitin sulfate A or C was first demonstrated to be the major mucopolysaccharide in normal human urine, but more recently heparitin sulfate, kertosulfate,

chondroitin sulfate B, and products of partial and complete desulfation of chondroitin sulfate A or C have been identified in normal human urine. The total amount of urinary mucopolysaccharides has also been shown to vary with age in children.

In this study the urinary excretion of mucopolysaccharides in Hurler's syndrome, their families, and normal man was examined. The chondroitin sulfates and total mucopolysaccharides were measured in urine specimens from humans of various ages and of both sexes. Mucopolysaccharides were also measured and identified in Hurler's syndrome and it was possible in some cases to measure the urinary mucopolysaccharides in families with affected children.

LITERATURE REVIEW

Chemistry and Metabolism of the Mucopolysaccharides

The mucopolysaccharides are linear polymers of high molecular weight. They usually consist of a disaccharide repeating unit of a hexosamine and a hexuronic acid moiety joined by a glycosidic bond. Currently eight distinct connective tissue acid mucopolysaccharides are known and Table IX gives a survey of the compounds and their components. Some recent reviews discuss their chemistry and biology (1,2,3).

Only two of the mucopolysaccharides found in connective tissue are non-sulfated, e.g., hyaluronic acid and chondroitin. Hyaluronic acid is widely distributed and has been extensively studied being the first mucopolysaccharide for which a complete structure was well-established. It has alternating glucuronic acid and glucosamine units. Chondroitin is less common in nature, and can be obtained by the removal of sulfate from the chondroitin sulfates. It has the same structure as hyaluronic acid except that it contains galactosamine instead of glucosamine.

Among the sulfated mucopolysaccharides, three types of chondroitin sulfates, A, B, and C, are recognized as

Table IX. Constituents of Acid Mucopolysaccharides

Mucopolysaccharide	Disaccharide Units	Linkage"
Hyaluronic acid	D-Glucuronic acid; N-acetyl-D-glucosamine	β, 1 3; β, 1 4
Chondroitin	D-Glucuronic acid; N-acetyl-D-galactosamine	β, 1 3; β, 1 4
Chondroitin sulfate A	D-Glucuronic acid; N-acetyl-D-galactosamine-4-sulfate	β, 1 3; β, 1 4
Chondroitin sulfate C	D-Glucuronic acid; N-acetyl-D-galactosamine-6-sulfate	β, 1 3; β, 1 4
Chondroitin sulfate B	D-Iduronic acid; N-acetyl-D-galactosamine-4-sulfate	α, 1 3; β, 1 4
Heparin*	D-Glucuronic acid-2-sulfate; N-sulfate-D-glucosamine-6-sulfate	α, 1 4; α, 1 4
Heparitin sulfate*	D-Glucuronic acid-2-sulfate; N-acetyl-D-glucosamine	α, 1 4; α, 1 4
Keratosulfate	D-Galactose; N-acetyl-D-glucosamine-6-sulfate	β, 1 4; β, 1 3

"The linkages are given in the same order as the names of the monosaccharide units.
*Possible structures. The exact structure is not known.

distinctive isomers and chondroitin sulfate-D had been isolated from shark cartilage, but its characterization is less firmly established. The repeating units of chondroitin sulfate-A are glucuronic acid and N-acetylgalactosamine-4-sulfate. Chondroitin sulfate-B, which is also referred to as β-heparin and dermatan sulfate, differs from A in that the glucuronic acid moiety is replaced by its C-5 epimer, L-iduronic acid. Chondroitin sulfate-C differs from A in that it is the 6-O-sulfate instead of the 4-O-sulfate.

The other sulfated mucopolysaccharides of connective tissue are heparin, heparitin sulfate, and kertosulfate. The characteristics of these compounds are not as firmly established as the chondroitin sulfates or hyaluronic acid. Heparin contains glucosamine, glucuronic acid and both O- and N-sulfate. Heparitin sulfate is probably related to heparin but appears to have less sulfate, especially N-sulfate. Keratosulfate is a polysaccharide that contains equal proportions of N-acetylglucosamine, galactose, and sulfate.

These mucopolysaccharides form an important part of the amorphous ground substance which lies between the extracellular fibers and the cells in connective tissue. Several biological functions have been described (cf. 3) for these compounds, such as calcification, control of electrolytes and water in extracellular fluids, wound healing, lubrication

of joints, blood coagulation, clearing activity, maintenance of the stable transport medium of the eye, and hair growth. Participation in most of these functions is undoubtedly due to the polyanionic nature of the compounds.

The general outline (cf. 3) of the biosynthesis of the mucopolysaccharides has been fairly well-established with the use of radioactive compounds and the discovery of the role of the nucleotide sugars in polysaccharide synthesis. The mechanism and extent of removal from tissues is still quite obscure. The rate of turnover of the various constituents of connective tissue varies. Collagen has a very slow turnover whereas the ground substance is relatively rapid. The half-life of hyaluronic acid as 2 to 4 days and chondroitin sulfate as 7 to 16 days has been calculated from isotopic studies of rabbit skin (4,5) and rat cartilage (6). The rate of turnover changes considerably with age (7). The half-life of keratosulfate appears to be very long and probably exceeds 120 days (8).

The removal of mucopolysaccharides from tissue may be accomplished both by extensive degradation and by excretion of the unchanged polymers. Skin extracts have been reported to promote the degradation of chondroitin sulfate B, but the mechanism of such breakdown is unknown (9). Reports have also appeared indicating the existence of hyaluronidase-like activities in mammalian tissues (10).

Mucopolysaccharides are also excreted in the urine (discussed in the next section). Kaplan and Meyer (11) studied the fate of injected chondroitin sulfates A, B, and C and heparitin sulfate in man and dog. After injection of chondroitin sulfate A and C there was a rapid disappearance from the blood but no significant amount was demonstrable in the urine. On injection of these mucopolysaccharides in heavier doses into a dog a small amount was recovered in the urine, but the blood level remained high for several hours. The fate of the unrecovered polysaccharides is unknown and it is difficult from this data to explain the normal excretion of chondroitin sulfate A. After injection of chondroitin sulfate B and heparitin sulfate they disappeared from the blood stream and a large percent could be recovered unchanged in the urine. On long continued injection of chondroitin sulfate B, a polysaccharide was excreted with the properties of heparitin sulfate.

Mucopolysaccharides in Normal Human Urine

In early studies, Astrup (12) precipitated a substance from urine with benzidine hydrochloride which was non-dialyzable, metachromatic with toluidine blue, alcohol-insoluble and possessed low anticoagulant activity. Kerby (13), using Astrup's method of precipitation, showed that the major component of the fraction migrated with commercial

chondroitin sulfate on paper chromatography and produced a single spot when mixed with the commercial chondroitin sulfate. Hamerman et al. (14) also found by paper chromatography an acidic substance migrating at a rate similar to that of chondroitin sulfate from the non-dialyzable urinary solids. DiFerrante and Rich (15), using quaternary ammonium salts as precipitant, were able to isolate sufficient amounts of urinary mucopolysaccharides for purification and identification. Their data concerning composition and properties indicated that the major mucopolysaccharide was most likely chondroitin sulfate A, but from their methods it was impossible to clearly differentiate between chondroitin sulfate A and C and minor constituents would not have been detected by their methods.

It soon became evident that chondroitin sulfate A was not the only mucopolysaccharide in normal human urine. From paper chromatography, paper electrophoresis, analytical and enzymatic data, DiFerrante (16) suggested that in addition to chondroitin sulfate A normal urine also contains hyaluronic acid. Heremans et al. (17) demonstrated by electrophoresis three different acid mucopolysaccharides in urine. The most abundant compound migrated at the same speed as commercial chondroitin sulfate. The other two peaks migrated at somewhat slower rates. King et al. (18) presented evidence for a glucosamine-containing acid mucopolysaccharide in normal human urine in addition to chondroitin

sulfate. From the presence of glucosamine, electrophoresis, and enzymatic data, they suggest that it is heparitin sulfate. Linker and Terry (19), using carbazole-orcinol color reactions for uronic acid, paper chromatography, and specific enzymatic hydrolysis, identified chondroitin sulfate A, chondroitin sulfate B, heparitin sulfate, and possibly a small amount of kertosulfate in human urine. The percent of each was estimated to be 80% chondroitin sulfate A, 10% chondroitin sulfate B, and 10% heparitin sulfate. DiFerrante (20), by fraction of urinary mucopolysaccharides on ECTEOLA cellulose and using enzymatic and chemical analysis of the peaks, identified chondroitin sulfate B, heparitin sulfate, and products of partial and complete desulfation of chondroitin sulfate A or C in addition to chondroitin sulfate A.

Kerby (13) also showed that the mean 24-hour excretion of mucopolysaccharides was 3.0 mg of glucuronic acid equivalent material in white females and 4.9 mg in white males. The difference between the two values was significant. DiFerrante and Rich (20) obtained slightly higher values using a different method for isolation. Their average per 24-hour urine collection was 3.77 mg of glucuronic acid material for women, 6.00 for men, 5.80 mg for girls (7-8 years), and 7.45 for boys (7-16 years). Since it was observed that the amount of mucopolysaccharides was higher in children, Rich et al. (21) investigated more thoroughly

the excretion in the urine of normal children. They found a gradual increase in mucopolysaccharide excretion during childhood, but a regular decrease occurred in the ratio of mucopolysaccharide excretion to creatinine excretion. Teller et al. (22) have also investigated urinary mucopolysaccharides in normal children and established the normal 95% probability range of mucopolysaccharide excretion in these children. Their results were similar to that described by Rich and co-workers with slightly lower values and they were unable to demonstrate a significant difference in excretion between girls and boys.

Mucopolysaccharide Metabolism in Hurler's Syndrome

Hurler's syndrome is a heritable disorder of a severe disturbance of mucopolysaccharide metabolism. Several names, such as Hurler's syndrome, gargoylism and lipochondrodystrophy, have been applied to this disorder. Several recent reviews have appeared (3,23,24,25).

Brante (26) was the first to suggest that the disorder was a mucopolysaccharidosis. By histochemical and isolation procedures he showed the presence in the tissues and urine of large quantities of a sulfated mucopolysaccharide which he believed to be a chondroitin sulfate. This was further clarified by Stacey and Barker (27) and Brown (28) who isolated a dextrorotatory sulfated

mucopolysaccharide which contained glucosamine from the liver of patients. Dorfman and Lorincz (29) and Meyer et al. (30) isolated and identified two sulfated mucopolysaccharides, chondroitin sulfate B and heparitin sulfate, from the urine of patients with Hurler's disease. Meyer et al. (31) later reported the presence and distribution of these two mucopolysaccharides in various organs of patients with Hurler's disease. The livers contained predominantly heparitin sulfate while in other organs, especially in the spleen, chondroitin sulfate B is the main polysaccharide. Since the identification of mucopolysaccharidosis as the defect in Hurler's syndrome, numerous procedures and methods have appeared for their determination in urine which appear to be of diagnostic value.

It soon became clear that there were several types of Hurler's patients. From genetic studies (cf. 24), there was identified a sex-linked and an autosomal type of inheritance. In the report by Meyer et al. (30), two cases of sex-linked and two cases of autosomal inheritance were reported and there was no obvious difference in the pattern of urinary mucopolysaccharides between the two groups. However, Terry and Linker (32) have distinguished four types by their mucopolysaccharide excretion pattern: classical autosomal recessive, classical sex-linked recessive, a type that excretes only heparitin sulfate, and an adult form. The autosomal recessive form was shown to

excrete significantly more chondroitin sulfate B in proportion to heparitin sulfate than the sex-linked cases.

Meyer et al. (30) also reported two cases with only heparitin sulfate in the urine, but they did not classify them as distinct types. Harris (33) published data from an analagous case in which the chemical study was performed by K. Meyer and suggested a possible new genotype of Hurler's syndrome. Sanfilippo et al. (34) confirmed these findings with eight patients with chemical, clinical, morphological and radiological studies. Maroteaux and Lamy (35) have recently presented a detailed study of this type. They observed seven patients that excreted only heparitin sulfate in the urine. They present clinical, radiological, genetic, and biochemical evidence and propose the term oligophrenia polydystrophy for this disorder. From the available data it would seem that this type is inherited as an autosomal recessive (32,35) and appears to differ clinically from the other types of Hurler's syndrome (35). Maroteaux et al. (36) have also studied a new type of Hurler's patient which excretes only chondroitin sulfate B in the urine.

MATERIALS AND METHODS

Mucopolysaccharides

Crude chondroitin sulfate and heparin were obtained from General Biochemical Incorporated. Chondroitin sulfate A was purified from the crude chondroitin sulfate by fractionation with cetyl pyridinium chloride and by column chromatography on Dowex-1-X2-Cl according to the method of Schiller et al. (37). It was further purified by alcohol fractionation as the calcium salt according to the method of Meyer et al. (38). Chondroitin sulfate B was a gift of Dr. Karl Meyer and chondroitin sulfate C was a gift of Dr. C. W. Castor. Hyaluronic acid was obtained from the Worthington Biochemical Company. Heparitin sulfate was a gift from the Upjohn Company.

Quantitative Measurements

Uronic acid determinations were performed by the carbazole method of Dische (39) with added borate (40) as modified by Bitter and Muir (41) and by the naphthoresorcinol method of Pelzer and Staib (42). Creatinine was determined by the method of Folin and Wu (43). Chondroitin sulfates were determined as previously described (44).

Urine Samples and Isolation of Mucopolysaccharide from Urine

Urine samples from patients with Hurler's syndrome were obtained from state schools for the mentally retarded in Michigan. Specimens were also obtained from three families each with an affected child. For the control studies, urine was obtained from the state schools for the mentally retarded and a local hospital. Controls were of both sexes and of various ages. The amount of urine for isolation of the mucopolysaccharides varied from 5 to 40 ml depending upon the availability, age, and whether from Hurler's patients or normal.

The mucopolysaccharides were isolated from urine by a slight modification of the method of DiFerrante and Rich (20) as previously described (45). Paper chromatography of mucopolysaccharides was carried out by the method of Spolter and Marx (46) employing the type III solvent system.

RESULTS

Mucopolysaccharide Excretion in Urine of Normal Man

There was some variation in the amount of mucopolysaccharides in different urine samples, but the data revealed a marked decrease in mucopolysaccharide excretion with advance in age in children and a close correlation with creatinine levels. A uniform excretion pattern becomes evident when the amount of chondroitin sulfates (Figure 6) or total mucopolysaccharides (Figure 7) per unit of creatinine are plotted against age. The marked decrease with age is apparent until the late teens after which the level of mucopolysaccharide per unit of creatinine remains constant. Total mucopolysaccharides per unit of creatinine varied from 60 to 140 in the very young and became constant at a value of about 6 in adults. The chondroitin sulfates per unit of creatinine varied from 50 to 80 in the very young and became constant at a value of about 2.5 in adults.

In addition to the decrease in the amount of mucopolysaccharide with age, a decrease in the chondroitin sulfate/mucopolysaccharide ratio is also apparent (Figure 8). This ratio varied from 0.60 to 0.80 in children to 0.30 to 0.50 in adults.

Figure 6. The amount of chondroitin sulfates (ChS) relative to creatinine in urine samples from humans of various ages.

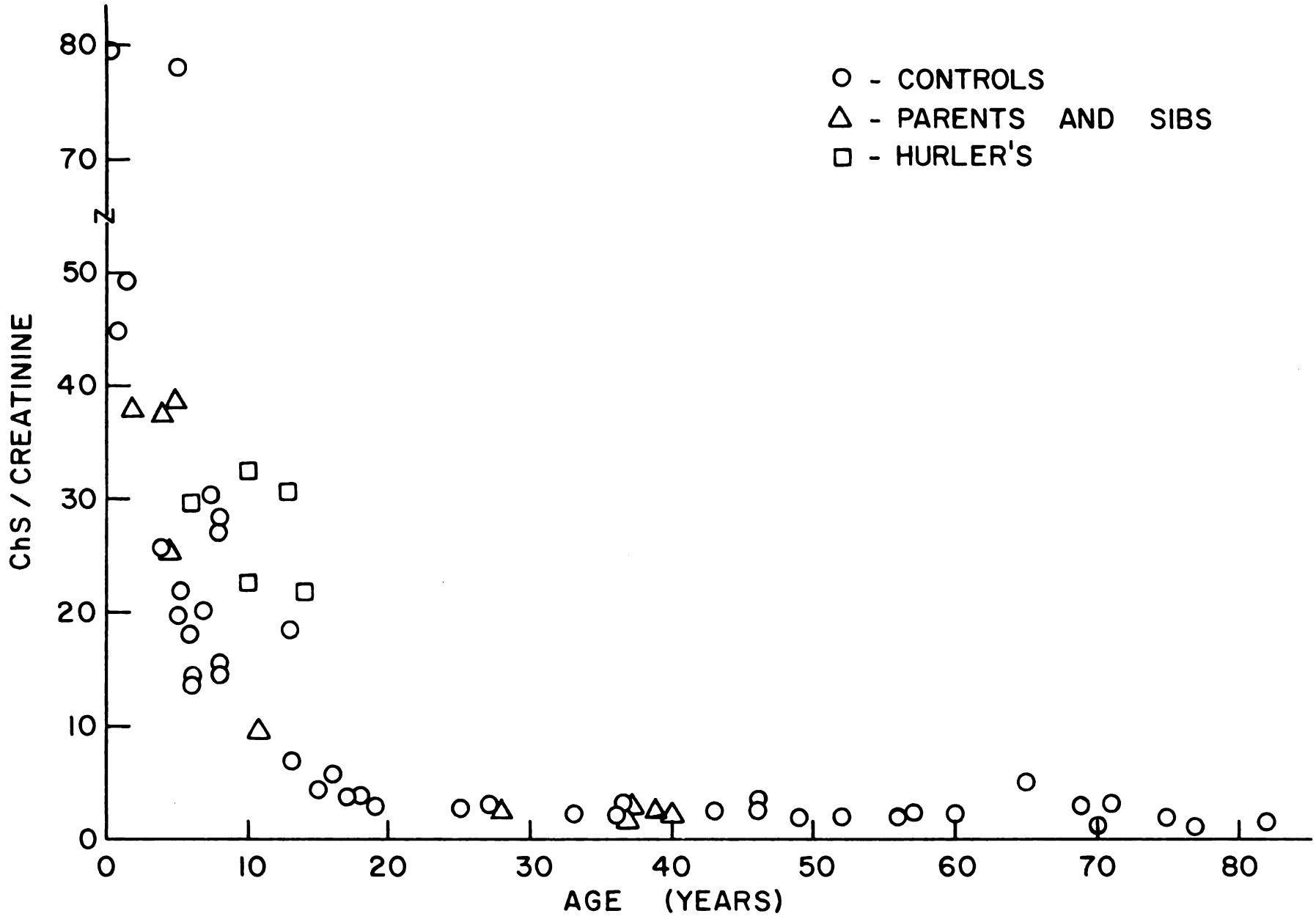

O - CONTROLS
△ - PARENTS AND SIBS
□ - HURLER'S
ChS / CREATININE
AGE (YEARS)
0
10
20
30
40
50
60
70
80

Figure 7. The total quantity of mucopolysaccharide (MPS) relative to creatinine in urine samples from humans of various ages.

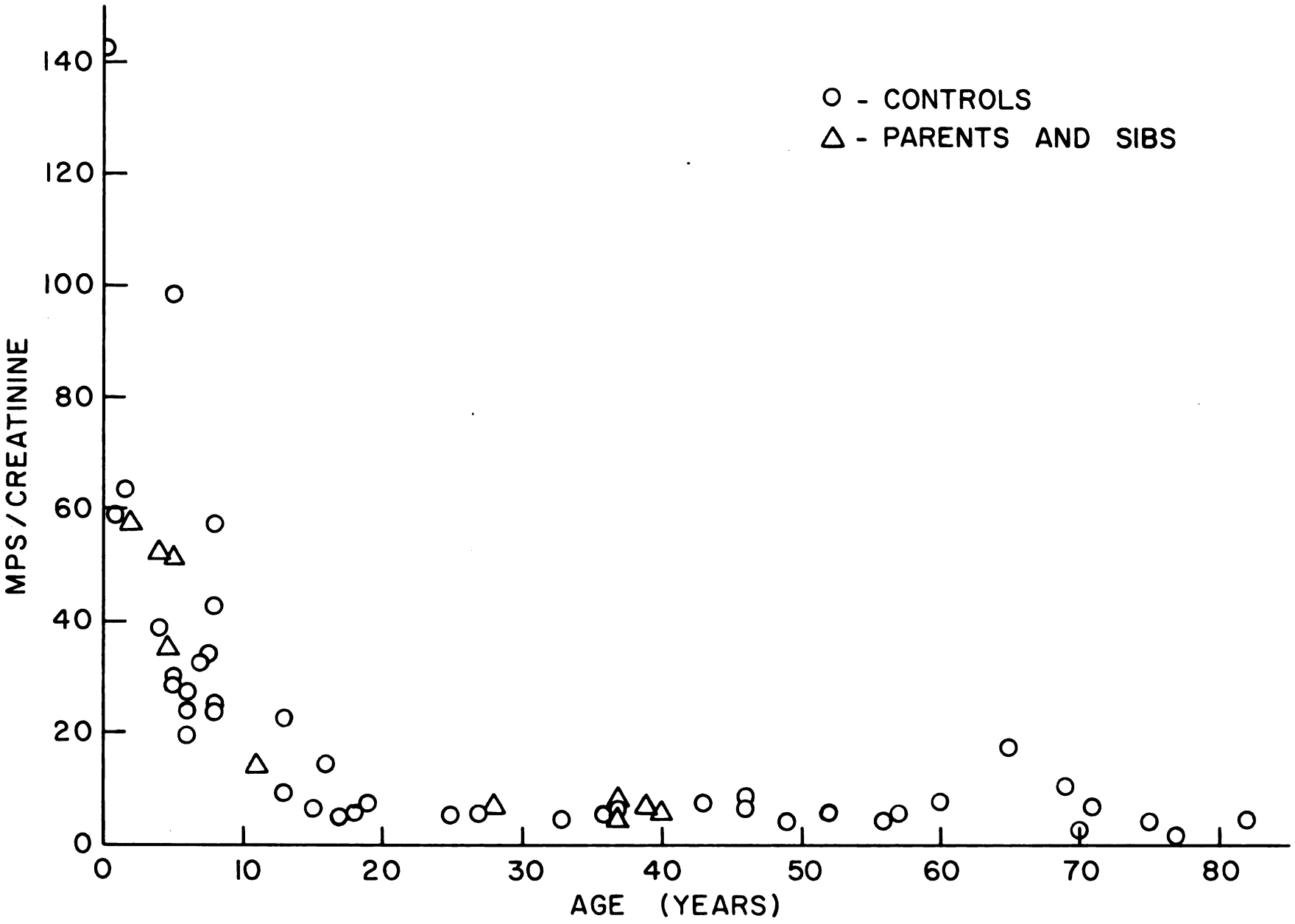
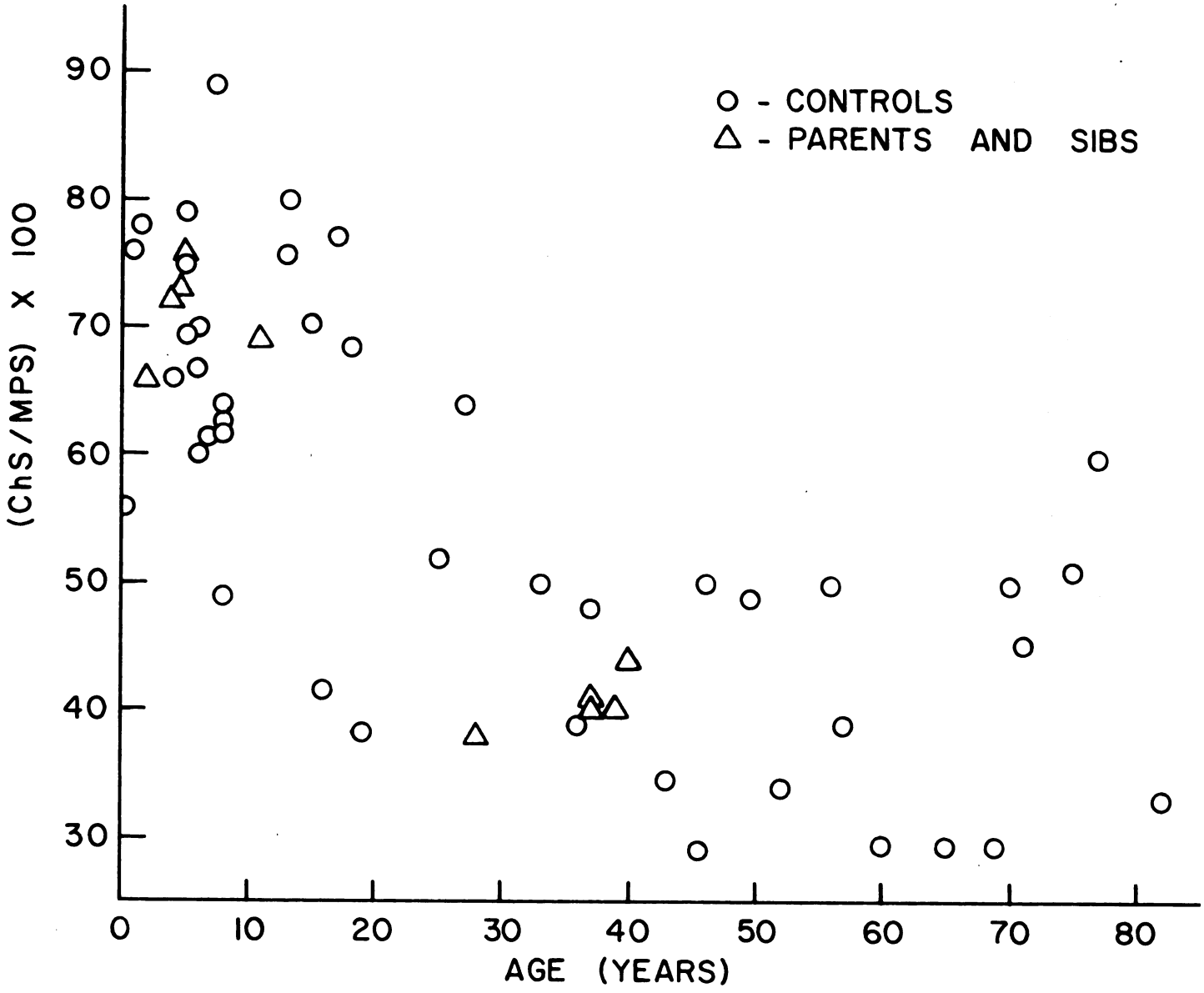

MPS / CREATININE
AGE (YEARS)
O - CONTROLS
Δ - PARENTS AND SIBS
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60
70
80

Figure 8. Ratio of chondroitin sulfates (ChS) to mucopolysaccharides (MPS) in urine samples from humans of various ages.


O - CONTROLS
Δ - PARENTS AND SIBS
(ChS/MPS) X 100
90
80
70
60
50
40
30
0
10
20
30
40
50
60
70
80
AGE (YEARS)

Mucopolysaccharides in Urine from Families with an Affected Child

Family 1 had three affected children and all excreted heparitin sulfate. Likewise the affected child in family 2 excreted hepartin sulfate. While the affected child in family 3 excreted both heparitin sulfate and chondroitin sulfate B. The data concerning mucopolysaccharides in urine samples from families with affected children are given in Table X. By all criteria employed the mucopolysaccharides in urine samples from all parents and siblings were within the normal range. When the samples from parents and siblings of affected patients are compared with the controls, the amount of chondroitin sulfates (Figure 1) and total mucopolysaccharides (Figure 2), and the chondroitin sulfate/mucopolysaccharide ratio (Figure 3) appeared to be in the same range.

Mucopolysaccharides in Urine from Patients with Hurler's Syndrome

The data on the mucopolysaccharides in urine samples from Hurler's syndrome are given in Table XI. Five of the six patients showed above normal C/N ratios, a normal amount of chondroitin sulfates, a high level of total mucopolysaccharides and a low chondroitin sulfate/mucopolysaccharide ratio. All these criteria would indicate that these patients excreted heparitin sulfate. The other patient excreted both chondroitin sulfate B and heparitin sulfate

Table X. Mucopolysaccharides in Urine from Parents and Siblings of Hurler's Syndrome

Subject	Relation	Age	C/N	Cr/ml	ChS/ml	ChS/Cr	MPS/ml	MPS/Cr	ChS/MPS
F.E.W.	Father of patients 1, 2, & 3	40	7.1	1.15	2.55	2.25	5.8	5.04	.44
M.F.W.	Mother of patients 1, 2, & 3	37	8.9	.525	.90	1.71	2.2	4.19	.41
F.A.W.	Brother of patients 1, 2, & 3	11	8.8	.625	6.0	9.6	8.7	13.9	.69
D.M.W.	Sister of patients 1, 2, & 3	4½	14.2	.93	23.7	25.5	32.6	35.1	.73
J.E.W.	Brother of patients 1, 2, & 3	2	12.8	.533	20.3	37.9	30.7	57.4	.66
D. W.	Father of patient 4	28	7.9	2.24	5.85	2.61	15.2	6.78	.38
R. W.	Brother of patient 4	5	13.0	.58	22.5	38.8	29.7	51.2	.76
D. S.	Father of patient 6	39	11.0	3.15	8.1	2.58	20.5	6.51	.40
M. S.	Mother of patient 6	37	8.9	.385	1.17	3.04	2.9	7.53	.40
D. S.	Sister of patient 6	4	9.7	1.62	60.7	37.5	84.7	52.3	.72

Abbreviations are the same as in Table XI.

Table XI. Mucopolysaccharides in Urine from Patients with Hurler's Syndrome

Patient	Age	Sex	C/N	Cr/ml	ChS/ml	ChS/Cr	MPS/ml	MPS/Cr	ChS/MPS	Paper Chromatography
1	10	M	19.9	1.34	34.0	25.4	248	185	13.7	ChS-A, HS
2	14	M	15.5	2.08	48.7	23.4	422	203	11.5	ChS-A, HS
3	13	F	13.4	1.11	34.7	31.3	334	301	10.4	ChS-A, HS
4	6	F	-	0.64	19.0	29.5	151	234	12.6	ChS-A, HS
5	10	M	8.0	0.62	21.5	35.0	259	421	8.3	ChS-A, HS
6	10	M	1.7	1.64	231.7	141.7	456	278	50.8	ChS-A, HS, ChS-B

Age is in years. C/N is the carbazole hexuronic acid/naphthoresorcinol hexuronic acid ratio. Creatinine/ml (Cr/ml) is given as mg/ml. Chondroitin sulfates/ml (Chs/ml) is expressed as μgm/ml and is based upon a purified sample of chondroitin sulfate A as standard in the chondroitinase assay (44). Total mucopolysaccharides/ml (MPS/ml) is expressed as μgm/ml and is based upon a purified sample of chondroitin sulfate A as standard in the carbazole hexuronic acid reaction (41).

as indicated by a low C/N ratio and excessive amounts of chondroitin sulfates and mucopolysaccharides.

On paper chromatography the one sample (No. 6) showed three spots with mobilities similar to chondroitin sulfate B, heparitin sulfate and chondroitin sulfate A. The other samples showed only two spots with mobilities similar to heparitin sulfate and chondroitin sulfate A. Our standard of heparitin sulfate streaked in this solvent system and it was not completely clear that the spots from the samples were heparitin sulfate. Judging from the mobility reported by Spolter and Marx (46), the chemical evidence, and the established fact that heparitin sulfate occurs in urine from Hurler's patients, it was concluded that the spots were heparitin sulfate. The solvent system for chromatography does not separate chondroitin sulfates A and C, but again it is well-established that chondroitin sulfate A is the major mucopolysaccharide in normal human urine.

DISCUSSION

In this study a marked decrease in the amount of total mucopolysaccharide per unit of creatinine was observed with age in children which is in general agreement with what has been published (21,22), but after the late teens the amount of mucopolysaccharides per unit of creatinine becomes constant with age. The amount of chondroitin sulfates in the urine of various age groups was also measured and a similar pattern to the total mucopolysaccharides was observed with age. A decrease in the ChS/MPS ratio with age was also observed which would indicate that a decrease in the amount of chondroitin sulfates relative to total mucopolysaccharides occurred with age. Therefore, the types of mucopolysaccharides at various ages as well as the total amount should be considered in control urine samples. A similar pattern of total mucopolysaccharide and chondroitin sulfates per unit of creatinine with age may occur in other mammals (45).

Abnormal excretion of mucopolysaccharides in the urine of heterozygous carriers of Hurler's syndrome has been seen by Teller *et al.* (47). They observed a low C/N ratio in suspected heterozygous carriers as compared to normal urine samples which would indicate an abnormal excretion of

chondroitin sulfate B. They also reported a normal mean C/Cr ratio in carriers which would indicate that the increase in total mucopolysaccharides was not detectably different from the normal. However, Linker and Terry (19), employing the C/N ratio and isolation of the mucopolysaccharides, observed no significant differences in urinary mucopolysaccharides in the carrier and normal individual.

In this study the mucopolysaccharides have been measured in urine samples from three families who have at least one child with Hurler's syndrome. In two families the affected children excreted heparitin sulfate and mucopolysaccharide excretion studies have not previously been reported in parents and siblings of this type of Hurler's syndrome. Since this defect is probably inherited as an autosomal recessive trait, the parents and possibly some of the siblings might excrete an excess of heparitin sulfate. The ChS/MPS ratio and total mucopolysaccharides appeared to be within the normal range. In the other family the affected child excreted both heparitin sulfate and chondroitin sulfate B. The mucopolysaccharides from the urine of both parents and a sibling showed a normal C/N ratio and normal amounts of mucopolysaccharides and chondroitin sulfates in the urine. In general, the mucopolysaccharides from urine of parents and siblings of Hurler's patients were within the normal range by all criteria. Possibly the methods are not sensitive enough to detect a very small amount of a given mucopolysaccharide, but

the parents and siblings do not appear to excrete a large amount of abnormal mucopolysaccharides.

In this survey of Hurler's syndrome in the state schools for the mentally retarded in Michigan, we have found that five out of six Hurler's patients excrete heparitin sulfate. Although, three of these patients were in the same family, still a large percentage of the patients excrete heparitin sulfate. Now that methods are available to distinguish the various types of Hurler's syndrome this latter type may be more prevalent than previously thought.

The determination of the normal excretion of mucopolysaccharides in urine is important because it may reflect the metabolism of these compounds in the body and the normal pattern is most likely used when comparing excretion in mucopolysaccharides disorders. The occurrence of mucopolysaccharides in normal human urine appears to be more complicated than originally observed. The chemical nature of the acid mucopolysaccharide normally present in urine is by no means settled, but it is clear that chondroitin sulfate B and heparitin sulfate occur in the urine in addition to the major mucopolysaccharide, chondroitin sulfate A.

SUMMARY

Mucopolysaccharides have been measured in urine samples of normal persons, patients with Hurler's syndrome and their relatives. A marked decrease was observed in the samples from controls in the amount of mucopolysaccharides and chondroitin sulfates per unit creatinine with age until the late teens after which the amount became constant. A decrease in the ChS/MPS ratio with age was also observed. After making appropriate allowances for age and creatinine, mucopolysaccharides excretion in parents and siblings of Hurler's syndrome was found to be similar to normal individuals by all criteria used. Several patients with the heparitin sulfate uria type of Hurler's syndrome were found in this survey.

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