Biochemical Genetics as Illustrated by Hereditary Galactosemia

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THE mere existence and biologic distribution of the sugar, galactose, is puzzling especially from the standpoint of an evolutionist. The difference between glucose and galactose is confined to the carbon atom number 4 (Fig. 1). The transition from glucoside to galactoside is essentially a kind of racemization between two configurations: the 4-hydroxyl in glucose occupies the equatorial position, whereas in galactose it occupies the axial position as related to the plane of the ring (Fig. 1).

It would be interesting to go deeper into the evolutionary aspects of galactose metabolism—the fact that the central nervous system and the milk gland have chosen to use galactose as one of their matrix elements. What could the positive vectors be? As will appear from the following discussion, galactose is not a necessary component of the diet of the newborn. Yet, we are not in a position to deny the possibility that given certain external influences the fitness of survival and reproduction may be influenced significantly by the presence of galactose in the diet. It is known, for instance, that the intestinal bacterial flora of the newborn animal is altered when lactose is replaced by sucrose. Nevertheless with only a few exceptions can we say that galactose is required by the living cell, insofar as this sugar can be made from glucose and glucose metabolites. As we shall see, we even have organisms for which the administration of galactose brings about serious disturbances.

Because of the complexity of galactose metabolism in higher animals, it is necessary to focus some attention on a particular chapter of carbohydrate metabolism and to introduce the various intermediary compounds which have been described in galactose metabolism. We are dealing here not only with free galactose or phosphorylated galactose but also with nucleotide hexoses, uridine-diphosphoglucose (URPPG) and uridine-diphosphogalactose (URPPGal). This important discovery we owe to Leloir.[1,2]

There are three major steps in the Leloir pathway, catalyzed by (1) Galactokinase: Gal + ATP $\rightarrow$ PGal + ADP; (2) Gal-1-P uridyl transferase: PGal + URPPG $\rightleftarrows$ PG + URPPGal; and (3) UDPGal 4-epimerase: URPPGal $\rightleftarrows$ URPPG.

The abbreviations used in this equation are meant to facilitate the understanding of mechanisms. Thus, galactose-1-phosphate is abbreviated to PGal in order to illustrate that the transferase catalyzes the "trading" of PG (glucose-1-phosphate) for PGal in the uridine nucleotide.

In animals, the enzymes catalyzing these reactions are largely or exclusively constitutive but unequally distributed among various tissues. In microorganisms, these enzymes are largely adaptive, i.e., they require galactose as inducer.

The system serves two functions: (1) to enroll galactose as an energy source in general carbohydrate metabolism, i.e., conversion to glucose-6-phosphate, triosephosphate or pyruvate; and (2) biosynthesis of more or less complex galactosides (and also polyglucosides).

As mentioned, most organisms, and certainly

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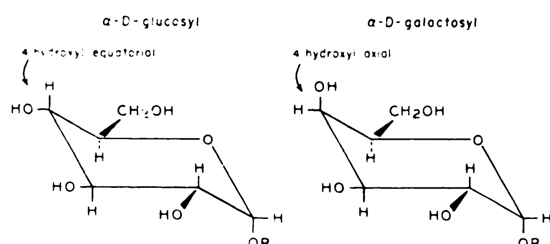

FIG. 1. Three-dimensional models of α-D-glucosyl and α-D-galactosyl compounds.

the higher animals, do not need any external supply of galactose for their growth and development. Whatever galactosides their tissues may contain can readily be made from glucose or from glucose metabolites through reaction 3, i.e., the 4-epimerase catalyzed reaction. A block in galactose metabolism is therefore apt to cause interference largely due to accumulation of intermediary products rather than on account of direct deficiency. As a general rule, jamming of intermediary products of a pathway is brought about by a block in any of the steps with the exception of the first one. This question will be dealt with more specifically later in this presentation.

CONGENITAL GALACTOSEMIA

An interesting type of a galactose-sensitive organism is the human subject afflicted with galactosemia. The disease in infants, congenital galactosemia, is characterized by specific tissue lesions (cataract, fatty degeneration in liver, retardation of brain function); by excretion of galactose in the urine; and, as shown by Schwarz and his co-workers,[3] by an accumulation of galactose-1-phosphate (Gal-1-P) in the red blood cells. These aberrations occur only if the affected subjects receive galactose. If a strictly galactose-free diet is imposed on the individual from birth, the organisms are practically insured against the development of specific galactosemic lesions. It is important here to bear in mind that in mammals the deposition of galactolipids in the central nervous system takes place exclusively after birth;[4] and yet, institution of a galactose-free diet provides practically complete protection against the development of cataract and feeblemindedness due to galactosemia.[5] This point is stressed here because it has been customary

TABLE I
Quantitative Comparison of Activities of Enzymes of Galactose Metabolism in Three Classes of Subjects

Subjects	Average Activities (μM conversion/hr./gm. cells, 37°C.)			
	Galactokinase	Gal-1-P Uridyl Transferase	UDP-Gal 4-Epimerase	PP-Uridyl Transferase
Nongalactosemic	0.10	4.8	0.32	10.0
Galactosemic	0.08	0.02	0.35	10.0
Galactosemic carriers	...	2.9	...	...

to refer to galactosemia as a block in 4-epimerase (Waldenase block). If this were so, postnatal galactose-free regimen would interfere with the deposition of brain galactolipids and, therefore, quite likely also with the development of normal intelligence.

An accumulation of Gal-1-P in the red cells of galactosemic subjects exposed to galactose might indicate the existence of an involved disturbance in the regulation of galactose metabolism somewhat analogous to diabetes mellitus, or alternatively a direct block in one of the enzymes of the Leloir system succeeding galactokinase. We preferred to focus our attention first on the enzyme Gal-1-P uridyl transferase, the existence of which had been predicted by Leloir[6] and which we observed a few years later in extracts of galactose-induced yeast.[7,8] We had developed specific enzymic methods[9,10] for characterizing most of these steps; we therefore considered ourselves reasonably well equipped to try our luck with the solution of the riddle of galactosemia. The enzymic technics permitted us to demonstrate that the defective enzyme in galactosemia is indeed Gal-1-P uridyl transferase; this was first demonstrated in lysates of erythrocytes (Table I). The other enzymes of the Leloir pathway were present.[11–13] Although this observation did clarify a number of problems, we were far from a solution since we had raised some new ones. Following are some of the problems which, to some extent, were clarified by our observation.

In view of the foregoing discussion on the postnatal deposition of galactose in the brain,

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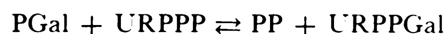
it is particularly noteworthy that 4-epimerase (Waldenase) is present in about the same amount in galactosemic subjects as in nongalactosemic subjects.

The transferase defect could be demonstrated in blood from the umbilical cord of newborn infants.[14] This concurs, of course, with the fact that the disease is an inborn error.

By the use of C^{14}-labeled galactose, we were likewise able to demonstrate the same enzyme defect in liver biopsy samples from galactosemic subjects.[14] In one case, we wondered whether we might have encountered an incomplete enzyme defect (a "leaky mutant," as the microbiologists call it). In this galactosemic adult small amounts of transferase were found in his liver tissue but none in his hemolysates. I am inclined to think a related transferase may have developed in this subject which can incorporate Gal-1-P in a somewhat different way into nucleotide. Earlier we had described the existence of such a transferase in galactose-adapted yeast.[7,8] This uridyl transferase catalyzes a reaction between PGal and uridine triphosphate (URPPP) according to the equation:



$$\text{PGal} + \text{URPPP} \rightleftarrows \text{PP} + \text{URPPGal}$$

This reaction is apparently predominant in plants.[15] Moreover, Isselbacher[16] found the alternative transferase in rat liver. Hence, it is quite conceivable that the alternative transferase is present in small amounts in the liver of human subjects and may increase in adults.

PARTIAL ENZYMATIC DEFECTS

If congenital galactosemia is a hereditary enzyme defect, one would expect to find partial defects in parents of galactosemic children (heterozygotes). The first approach to this problem was made by Holzel and Komrower in 1955.[17] They used the galactose tolerance test as a criterion and found that either one or both the parents of galactosemic children had lower values in the tolerance test. The identification of one specific enzymic defect—the Gal-1-P uridyl transferase defect—encouraged us to try to devise a specific test for the detection of heterozygous carriers.

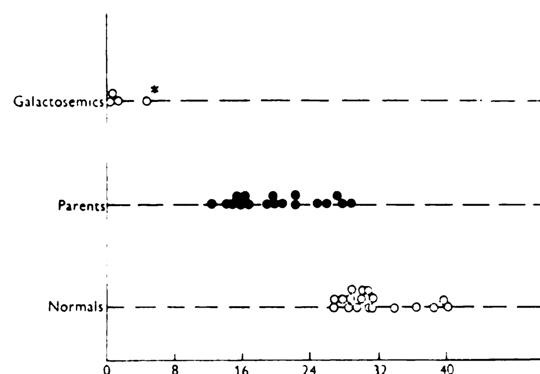


FIG. 2. The activity of Gal-1-P uridyl transferase expressed as μl. O_2 consumed per hour by 0.3 gm. of red cells from three different classes of subjects. (1) Nongalactosemic (mainly normal healthy persons); (2) persons suspected of being heterozygous with respect to the gene for the synthesis of the transferase since they have offspring with congenital galactosemia; and (3) corresponding homozygous affected persons, i.e., galactosemic. From: KIRKMAN, H. M. and BYNUM, E. *Ann. Human Genet.*, 23: 117, 1959.[18]

In order to make a technic available for this purpose, it would be most desirable to have a method with a built-in control which ensures that one is measuring rates in the range of zero order kinetics. Kirkman[18] used a very simple device, the old Barron-Engel'gardt-Warburg manometric determination of oxygen consumption in hemolysates (brought about by addition of methylene blue and metabolites). This method was so "tailored" as to fit the purpose of comparing transferase activities between normal subjects and suspected galactosemic carriers; it scores more than 20 points of measurements with high accuracy. The details of the method have been described elsewhere.[18]

The manometric assay showed[18,19] that about 80 per cent of the parents of the galactosemic subjects who were surveyed had enzyme titers which were more than 2 standard deviations below the mean of nongalactosemic subjects (Fig. 2). Kirkman is inclined to believe that if hemolysates from normal subjects, as well as hemolysates from brothers and sisters of suspected carriers, were assayed the manometric assay might identify practically all suspected carriers individually. To our knowledge, this is the first time that a method for measuring a partial enzyme defect stands a

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chance of being used for such purposes, i.e., consultations of parents in matters concerning eugenics and preventive medicine.* With reference to the latter aspect, from Kirkman's distribution curve (Fig. 2) it will be evident that an appreciable fraction of carriers show enzyme titers below 50 per cent of the average of normal titers. This is of great importance in pregnancy, during which state the galactose tolerance is somewhat lowered for other reasons. If a pregnant mother, heterozygous with respect to the gene directing the synthesis of Gal-1-P uridyl transferase, carries a homozygous galactosemic fetus, excessive milk drinking during her pregnancy may expose the fetus to hazardous amounts of galactose. In a fraction of such cases an identification of the enzyme defect in the umbilical cord blood, such as we described earlier,[14] may therefore not be of much use with respect to dietary preventive measures. The preventive measures should have been used on the mother as well.

The incidence of hereditary galactosemia is not known. We have had thirty-five to forty cases under investigation: in all of our cases the transferase was affected; the other enzymes of the Leloir pathway were found to be unaffected. Our clinical colleagues can report numerous cases in which the disease was diagnosed incorrectly, the enzymic test established the true nature of the disease.[20]

That early diagnosis and institution of galactose-free diet is crucial is too well known a fact to warrant an elaboration here. If this is not done, permanent cataract, intelligence defects and liver cirrhosis will ensue. The biochemical basis for the tissue damage is unknown as yet. The discovery by Schwarz and his associates[3] that Gal-1-P accumulates in the erythrocytes of galactosemic subjects placed the spotlight on a most interesting problem: it has been found[21,22] that Gal-1-P inhibits phosphoglucomutase, i.e., the enzyme which catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate. As emphasized, especially by Najjar,[23] it seems worth considering this inhibition as being responsible for the development of some of the tissue damage characteristic in galactosemia.

In order to learn more about the genotypic and phenotypic characteristics of hereditary blocks in Gal-1-P uridyl transferase we have extended our search to microorganisms. It has been known for some time, mainly through the work of the Lederbergs,[24,25,28] that certain K_{12} strains of Escherichia coli are unable to metabolize galactose and are also unable to grow on galactose as the sole carbon source,

* A few methodologic problems which need clarification are discussed briefly here. Kirkman emphasizes that the manometric method gives about 50 per cent of the maximum values which can be obtained by the two-step enzymic method designed for determining initial rates. This design was performed as follows:[34] Small amounts of hemolysates (0.05 ml. of packed erythrocytes) were incubated at 37°c. for brief periods of time (e.g., five and ten minutes) with relatively high concentrations of reactants (0.17 μM of URPPG and 0.85 μM of Gal-1-P were used). If Tris buffer is used the rates of transferase in hemolysates from normal subjects (or more correctly, homozygous nongalactosemic subjects) ranged from 3.5 to 5.8 μM/gm. cells hour.[34] In the presence of phosphate buffer (either at pH 7.3 or pH 8) the rates are consistently cut down to half. It is quite possible by the modified two-step enzymic method to find lowered rates in hemolysates from heterozygous carriers of galactosemia; we found values as low as 1 to 1.5 μM.[34] By the use of an almost identical set-up, Brettauer and his co-workers[36] recently obtained the following values: nongalactosemic subjects, 4.8 to 8.1 μM; carriers of galactosemia, 2.9 to 3.7 μM. These values are somewhat higher than ours. One may logically ask why not use the enzymic technic just outlined as the method in population studies, especially since the manometric method is best reproduced in phosphate buffer at pH 7.3, conditions which permit only half of the maximum rates which can be found in Tris buffer?

The reason for our choice of the manometric method is that it shows the investigator whether he is measuring initial rates (zero order kinetics) or not, and this is the essential point in comparing enzyme rates from homozygotes and heterozygotes. The enzymic method as used by Brettauer and his co-workers,[36] i.e., scoring 1 point, does not give such a guarantee and should, therefore, not be used as the sole method "to detect parents who are likely to give birth to galactosemics."[36] Hsia et al.[37] failed to provide convincing evidence for enzyme defects in heterozygotes because they used, against our warnings, a diagnostic method which we had designed exclusively for detecting galactosemic patients.[4] It is obvious that considerations of simple kinetics must be given due respect when partial enzyme defects in heterozygotes are under study. The manometric method would in general be the reliable guide at the present time.

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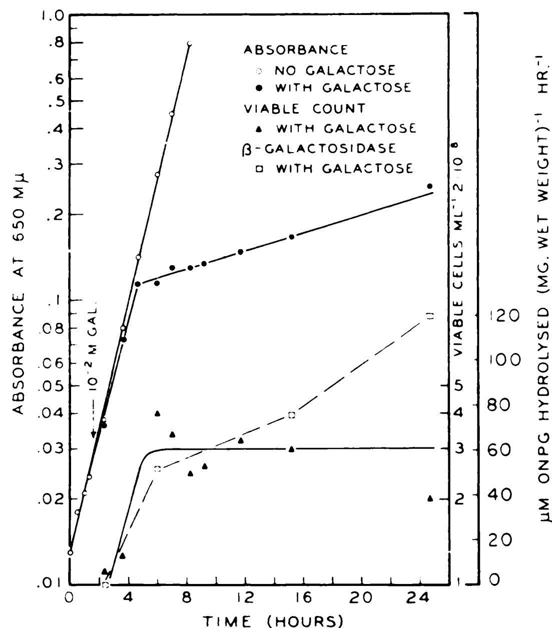

FIG. 3. Growth and division of E. coli K_{12} strain 3104 at 37°c. under aerobic conditions. Growth as well as viable counts plotted logarithmically. The activity of β-galactosidase (on a cellular basis) is plotted linearly and expressed as micromoles of orthonitrophenyl galactoside hydrolyzed per hour per micrograms toluenized cells (wet weight). The β-galactosidase activity of the cells which were grown in medium without galactose was too low to record on this graph (less than 1 μM/mg./hour). The β-galactosidase activity in the medium of nontoluenized cells grown with or without galactose is negligible (less than 1 μM/mg./hour). From: YARMOLINSKY, M., KALCKAR, H. M., WIESMEYER, H. and JORDAN, E. *Proc. Nat. Acad. Sc.*, 45: 1786, 1959.[31]

so-called gal-mutants.[24,25] The many gal-mutants had so far only been characterized genotypically. The challenge was to try to characterize them phenotypically and more specifically with respect to the supposedly primary gene products, the enzymes.

Kurahashi[26] in our group first undertook to apply the enzymic technics used in the identification of the defect in hereditary galactosemia to bacterial mutants. It soon became apparent that there were at least four genotypically different mutants which were phenotypically identical, i.e., they all had a practically complete defect in transferase. Likewise, there were three different mutants (different "mutons" in same "cistron"[27]) which phenotypically could be characterized as "galactokinase-less." Finally, there were various types of single mutants[28] "leaky" with respect to the two enzymes mentioned (i.e., greatly lowered activity as compared with the wild type) and more or less completely defective with respect to the 4-epimerase.[29]

Characteristic for the genotypes, which are unable to synthesize Gal-1-P uridyl transferase, is a phenomenon which we call "galactose-induced sensitivity" because the cells react like cells in an unbalanced state only when galactose permease and galactokinase are induced.[26,29,30] Under these conditions, Gal-1-P accumulates in large amounts just as in the afflicted children referred to previously. In most of the E. coli strains with which we have been working (Lederberg's K_{12} galactose mutants), the abnormal state could be called "galactose-induced bacteriostasis" because the cells do not lyse and remain viable[30] (see Fig. 3). The lack of lysis is borne out not only by the fact that turbidity and viable cell count remain constant but also by the fact that the medium does not contain more than traces of enzymes such as β-galactosidase. This should be emphasized because Fukasawa and Nikaido[31a] have recently described galactose-negative mutants in which induction by galactose brings about lysis and death, unless the cells are grown in hypertonic medium (*cf.* also Ushiba and Kitasato[31b]) in which case cells without cell walls (protoplasts) are formed. We have shown,[29] as well as Fukasawa and Nikaido,[32] that these "galacto-lytic" strains have a single defect in 4-epimerase.

The galactose-sensitive strains (either galactostatic or galactolytic) can undergo mutation. If grown in glycerol ammonia mineral medium in the presence of galactose, there is a strong selection either for reversions to galactose-positives or further mutation to galactose-negatives with an additional block early in the pathway. In this way we have obtained double mutants.

The relevance of these microbiologic studies to the problems of galactosemia in man is obvious. In the transferase-less bacterial mutants galactose-1-phosphate accumulates during the induced galactostasis; UDPGlucose levels tend to go down. In the galactolytic

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state, as well as just prior to this state, there is a striking accumulation of UDPGalactose.[33]

The nature of the genetic block in terms of protein (i.e., absence of protein or production of proteins which crossreact with antibodies against transferase but are catalytically inactive) is under study.

At the present time several groups in America have had success in cultivating skin fibroblasts from human subjects. I have voiced the opinion that hereditary galactosemia at present is one of the few inborn errors of metabolism which can be studied in tissue cultures.[34] This is due to the following circumstances: (1) Galactosemia has been identified as a defect in a particular enzyme which is relatively well defined, transferase (Gal-1-P/G-1-P uridyl transferase). (2) The transferase defect is not confined to a single type of cell but has been demonstrated in any cell type which shows galactose metabolism. (3) Mutations to reversions or transformations with DNA from transferase-negative to transferase-positive might be readily detected and selected because of the toxic effect of galactose toward the transferase-negative cells.

Krooth and Weinberg[35] have recently been able to demonstrate the defect in galactose metabolism in fibroblast cultures from galactosemic subjects. Such cultures are unable to use galactose as a hexose source and unable to form carbon dioxide from galactose. The latter study was performed with C^{14}-labeled galactose, collecting radioactive carbon dioxide. This seems to constitute a highly sensitive method. In our laboratory we have found a defect of transferase in sonicates from fibroblast cultures from galactosemic subjects. Enzyme studies on galactosemia in tissue cultures are only the beginning. Enzyme defects in glucose-6-phosphate dehydrogenase or in phosphatase should lend themselves, too, to fundamental studies.

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