Galactose Conversion to D-Xylulose: An Alternate Route of Galactose Metabolism

Abstract. *Galactose dehydrogenase, a soluble enzyme of the mammalian liver, catalyzes the conversion of galactose to galactonic acid. This reaction, together with the subsequent formation of 3-ketogalactonic acid which can be decarboxylated to yield* D-*xylulose, is a newly discovered pathway for the metabolism of galactose. This pathway may account for the oxidation of galactose observed in patients with galactosemia.*

The oxidation of galactose by mammalian cells is currently thought to proceed almost exclusively through the following reactions (*1*)

$$\text{Gal} + \text{ATP} \rightleftharpoons \text{Gal-1-P} + \text{ADP}$$
$$\text{Gal-1-P} = \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal}$$
$$\text{UDPGal} \rightleftharpoons \text{UDPG}$$
$$\text{UDPG} + \text{PP} \rightleftharpoons \text{G-1-P} + \text{UTP}$$

catalyzed by the enzymes galactokinase, Gal-1-P uridyltransferase, UDPGal-4-epimerase, and UDPG pyrophosphorylase, respectively (*2*). We now report evidence for the existence in mammalian liver of a new pathway (Fig. 1). This pathway has none of the intermediates of the conventional pathway, does not involve phosphorylated or nucleotide-linked intermediates, and is able to convert galactose to a form which can be used in the pentose phosphate shunt.

The first enzyme, galactose dehydrogenase, oxidizes the aldehyde group of galactose in the presence of NAD, and its activity can be found in crude preparations of liver from rat, mouse, dog, hamster, guinea pig, rabbit, monkey, and man. Initial velocity measurements are obtained by monitoring the reduction of NAD spectrophotometrically. Routine assays contain 100 μmole of galactose, 3 μmole of NAD, 40 μmole of tris (*p*H 8.6), and 0.2 to 0.4 mg of protein in a final volume of 1 ml. In rat liver the activities of galactose dehydrogenase and galactokinase are of similar magnitude; 10 mμmole of galactose is oxidized or phosphorylated per minute per milligram of supernatant protein when each enzyme is assayed in its optimum conditions (*3*). Ninety percent of the activity is present in the soluble cellular fraction (the supernatant from homogenates centrifuged in 0.25*M* sucrose at 100,000*g*); this preparation has no glucose dehydrogenase activity. The remaining 10 percent is found in the microsomal fraction which requires treatment with high-frequency sound for solubilization of the enzyme. Preparations of testis, kidney, gut, and mammary gland have minimal activity (0.5 to 2.0 mμmole of galactose oxidized per minute per milligram of supernatant protein), but none is present in preparations of muscle, spleen, brain, or lens, or in red and white blood cells. Galactose dehydrogenase can be purified 100-fold from Sprague-Dawley rat liver. Ion exchange resins can not be used in the purification since they irreversibly inactivate the enzyme. The *p*H optimum of enzyme activity is 8.2, and the K_m (Michaelis-Menten constant) for galactose is 0.03*M* and for NAD $2.5 \times 10^{-4}M$; NADP is 5 percent as effective as NAD.

Identification of the reaction product is complicated by marked inhibition of enzyme activity by the reduced NAD generated during the reaction (detectable at $5 \times 10^{-5}M$), but large amounts of the products are formed if reduced NAD is recycled (*4*). When a preparation containing 100 μmole of galactose-1-C^{14}, 0.5 μmole of NAD, 20 μmole of tris (*p*H 8.6), and 40 μg of purified enzyme is incubated for 4 hours at 37°C and passed through a Dowex-1 acetate column, a radioactive fraction can be eluted with 0.1*M* ammonium acetate (*p*H 5.4). The eluant sugar migrates like authentic galactonate (*5*) when it is subjected to high-voltage electrophoresis and three paper chromatographic systems. When the eluant sugar is heated for 5 minutes in 1*N* HCl, lactonization occurs, and subsequent migration is identical to that of galactono-γ-lactone upon electrophoresis and paper chromatography. A large incubation mixture containing nonradioactive galactose can be similarly treated, and a sugar acid can be precipitated with dioxane. This is identified as galactonic acid by chromatography; the sugar acid does not react with the usual sprays used to detect phosphorus, aldehyde and keto groups, uronic acids, and pentoses.

The immediate conversion product of galactose by galactose dehydrogenase is galactonolactone which, under the alkaline conditions of the assay, is spontaneously converted to galactonate. The hydroxamic acid derivative is trapped in large amounts when the incubation is carried out at *p*H 6.9 in the presence of neutral hydroxylamine (*5*). Stoichiometric studies reveal that the disappearance of galactose is accounted for by the appearance of galactonolactone at neutral *p*H and of galactonate at alkaline

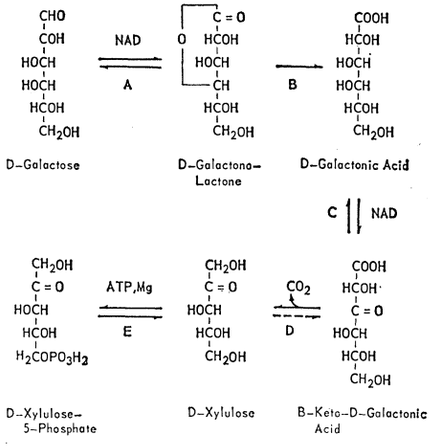

Fig. 1. Proposed scheme of alternate pathway of galactose metabolism by mammalian liver. Enyzmes are: *A*, galactose dehydrogenase; *B*, lactonase; *C*, β-L-hydroxy acid dehydrogenase; and *E*, D-xylulokinase. Decarboxylation (*D*) is probably enzymatic.

*p*H. Rat liver has a high activity of a soluble enzyme which acts on galactonolactone to yield galactonate (Fig. 2). In 15 minutes, 18.7 μmole of lactone disappear and 16.5 μmole of galactonate appear when 50 μl of the crude preparation is used. This delactonizing reaction is irreversible.

The further metabolism of the galactonic acid which is formed (steps *C* and *D*, Fig. 1) was studied by measuring the $C^{14}O_2$ evolved after incubation of C^{14}-galactonate with soluble fractions from rat liver homogenates. These studies suggest the presence of a NAD-linked decarboxylation at carbon No. 1 (Table 1). Addition of nonradioactive galactose decreases the $C^{14}O_2$ yield from galactonate-1-C^{14}; this suggests that the galactose is being converted to galacto-

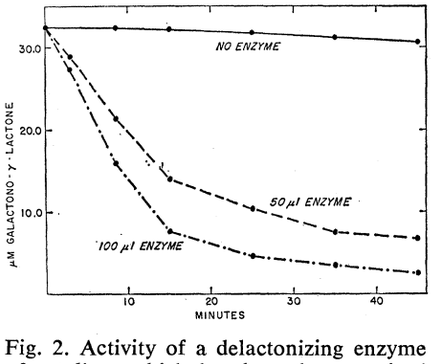

Fig. 2. Activity of a delactonizing enzyme of rat liver which has been homogenized with 5 volumes of 0.05*M* sodium phosphate buffer (*p*H 7.0) and centrifuged for 1 hour at 32,000*g*. The incubation medium contains 33 μmole galactono-γ-lactone (*5*). 100 μmole tris (*p*H 7.8), and 50 or 100 μl of liver supernatant (20 mg of protein per milliliter) in a final volume of 1.0 ml and *p*H 7.0.

Table 1. Liberation of $C^{14}O_2$ from C^{14}-galactonate by the supernatant of rat liver homogenized with five volumes 0.1*M* sodium phosphate (*p*H 7.0) centrifuged at 32,000g and dialyzed for 24 hours against the same buffer. Incubation medium consists of 3 μmole of galactonate-1-C^{14} (except for last sample which has galactonate-2-C^{14}) of specific activity 0.11 μc/μmole, 20 μmole tris (*p*H 8.6), and 10 mg of protein in a final volume of 1.0 ml, *p*H 7.8. Other additions as indicated are NAD or NADP, 3 μmole; ATP, 6 μmole; $MgCl_2$ 3 μmole; and galactose, 100 μmole per milliliter. Incubation is at 37°C for 2 hours, and $C^{14}O_2$ is collected in hyamine and counted by liquid scintillation spectrometry (*6*).

Additions	$C^{14}O_2$ liberated (count/min)
None	92
NAD	2660
NADP	157
NAD + NADP	2592
ATP + Mg^{++}	148
NAD + NADP + ATP + Mg^{++}	2288
NAD + galactose	452
NAD (galactonate-2-C^{14})	81

nate. This is confirmed by the observation that these enzyme preparations cause evolution of $C^{14}O_2$ from galactose-1-C^{14} (when NAD is added) but not from galactose-2-C^{14}; evolution of $C^{14}O_2$ can be decreased by adding nonradioactive galactonate. Kidney, brain, gut, and testis preparations also convert galactonate-1-C^{14} to $C^{14}O_2$, even though the activity of galactose dehydrogenase is very low or absent in these tissues. White and red blood cells are inactive in the decarboxylation reaction.

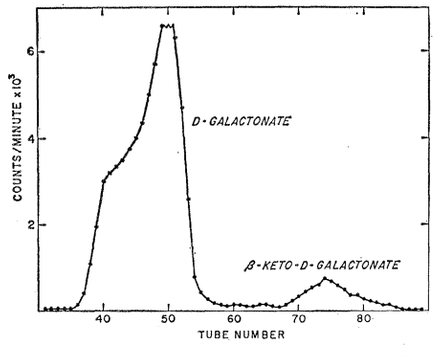

Fig. 3. Dowex-1 acetate chromatography of an incubation mixture which contains 15 μmole of NAD, 100 μmole tris (*p*H 8.6), 30 μmole galactonate-1-C^{14}, 0.1 mg of NADH oxidase (*5*), and 30 mg of the liver enzyme preparation (described in Table 1) in a final volume of 4.0 ml. Incubation is at 37°C for 1 hour. The column size is 15 by 1 cm, and elution is performed with a two-chambered continuous gradient (90 ml of 0.005*M* ammonium acetate against 90 ml of 0.500*M* ammonium acetate *p*H 7.0). Each collection is 1.5 ml.

The product of decarboxylation can be isolated and identified as D-xylulose (*7*) by paper chromatography and by its characteristic reaction with cysteine carbazole. The enzyme responsible appears to be similar to the one purified from hog kidney by Smiley and Ashwell, which they called β-L-hydroxy acid dehydrogenase because of its wide substrate specificity (*8*). An unstable intermediate in the conversion of D-galactonate to D-xylulose, presumably β-keto-D-galactonate, can be isolated in a manner similar to that used by Smiley and Ashwell in the identification of their intermediate (Fig. 3).

When β-keto-galactonate (tubes 69 to 80, Fig. 3) is boiled for 1 minute, it loses its radioactivity (as $C^{14}O_2$) and D-xylulose is then detectable by the cysteine carbazole reaction. Whether the decarboxylation in vivo (step *D*, Fig. 1) is spontaneous or is catalyzed by an enzyme cannot be determined at present.

The D-xylulose can be readily phosphorylated by a liver D-xylulokinase (*9*) to D-xylulose-5-phosphate. Pentose phosphate can then be converted to glucose and its phosphorylated derivatives. In this way the oxidative fate of galactose in this pathway resembles that of galactose in the conventional pathway.

Patients with congenital galactosemia, who lack gal-1-P uridyltransferase, are able, nevertheless, to oxidize some galactose (*10*), and in certain cases the oxidation of galactose-1-C^{14} (to $C^{14}O_2$) is nearly normal (*11*). It has been suggested that the liver enzyme UDPGal pyrophosphorylase may act to bypass the defective step and hence account for the galactose metabolism (*12*). However, the activity of this enzyme in liver supernatant preparations is at least 4000 times less than that of galactose dehydrogenase (0.0024 mμmole of galactose utilized per minute per milligram of protein at 38°C compared to 10 mμmole/min per milligram of protein at 20°C). It is reasonable to suspect that the "galactonic acid" pathway may account for the residual galactose metabolism in galactosemic individuals. By this pathway carbon No. 1 of galactose is removed directly as CO_2 whereas carbon No. 2 is more indirectly converted to CO_2 (via the pentose shunt). We compared the oxidation of 1 g of intravenously administered galactose-1-C^{14} and of galactose-2-C^{14} to expired $C^{14}O_2$ (5 μc in normals, 2 μc in galactosemics) in normal persons and in patients with congenital galactosemia. In normal volunteers the curves for the excretion of $C^{14}O_2$ obtained with both substrates are identical. However, in three Caucasian galactosemics with diminished ability to oxidize galactose, the excretion of labeled CO_2 from galactose-1-C^{14} is much greater than that from the C-2-labeled sugar. These results suggest that in normal subjects much of the galactose which is given is metabolized to glucose via the conventional pathway since the curves of expired CO_2 resulting from administration of C-1- and C-2-labeled glucose are nearly the same. In galactosemic individuals, the differences between the excretion curve obtained when galactose-1-C^{14} is given and that when galactose-2-C^{14} is given suggest that much of the residual galactose oxidation proceeds by the "galactonic acid" pathway.

Although galactose dehydrogenase has been described in *Pseudomonas saccharophila* (*13*), it has not been previously described in mammals. The product of galactose dehydrogenation in the bacterium is also galactonic acid, but its further metabolism is different; a 2-keto-3-deoxy intermediate is formed before the molecule is split. Furthermore, it appears that the formation of 2-keto-3-deoxy sugar acids is a general metabolic mechanism in the bacterial utilization of sugars (*14*). Such intermediates have not yet been described for mammals. Also, 3-keto sugar acid derivatives of the type described here for D-galactonic acid and by Smiley and Ashwell for L-gulonic acid (*8*) have not yet been reported in bacteria and may be unique to mammalian systems.

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References and Notes

1. H. M. Kalckar, *Science* **150**, 305 (1966).
2. Abbreviations: Gal, galactose; ATP, adenosine triphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; Gal-1-P, galactose-1-phosphate; G-1-P, glucose-1-phosphate; UDPG, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose; PP, inorganic pyrophosphate; NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; tris, tris (hydroxymethyl)aminomethane.
3. P. Cuatrecasas and S. Segal, *J. Biol. Chem.* **240**, 2382 (1965).
4. Modified from T. Akazawa and E. E. Conn, *ibid.* **232**, 403 (1958). The reduced NAD which is formed can be rapidly reoxidized by adding 0.01 μmole of resorcinol, 5 μmole of hydrogen peroxide, and 10 μg of purified horseradish peroxidase per milliliter of incubation mixture; reduced NAD and resorcinol are recycled, and only the product of galactose oxidation accumulates.
5. Potassium D-galactonate was prepared by the method of S. Moore and K. P. Link, *ibid.* **133**, 293 (1940); D-xylulose was a gift of Dr. G. Ashwell, NIH; purified NADH oxidase (*Clostridium Strichlandii*) was provided by Dr. T. C. Stadtman, NIH; lactones were determined by the method of S. J. Hestrin, *ibid.* **180**, 249 (1949); galactonic acid was assayed by the same method after it was boiled in 1N HCl for 5 minutes; galactose

was assayed by the specific enzymatic method of G. Avigad, D. Amaral, C. Asensio, B. L. Horecker, *ibid.* **237**, 2736 (1962).
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Preferential Mating versus Mimicry: Disruptive Selection and Sex-limited Dimorphism in Papilio glaucus

Abstract. *Spermatophore counts in wild females of* Papilio glaucus *show that the monomorphic nonmimetic male mates less frequently with the mimetic female morph than with the nonmimetic female morph. Female dimorphism in this species cannot be maintained by heterozygous advantage. Mating preference for the nonmimetic female may sufficiently counteract avian predation pressure favoring the mimetic female to account for the maintenance of the nonmimetic morph in the population in those areas in which the model is abundant and to account for the reduction in frequency or elimination of the mimetic morph in those areas in which the model is less numerous or absent.*

Sex-limited dimorphism exists in many populations of *Papilio glaucus* Linnaeus, the eastern tiger swallowtail butterfly of North America. Males are striped black on yellow; females are like males or are predominantly black (Fig. 1). The dark female morph mimics *Battus philenor* (Linnaeus), a swallowtail unpalatable to avian predators (*1*).

The occurrence of the mimic (dark morph of *P. glaucus*) is correlated with the distribution and abundance of the model (*B. philenor*). *P. glaucus* ranges from Newfoundland to Alaska, eastern British Columbia, the western Dakotas, and western Nebraska, and thence throughout the eastern United States, mostly east of the hundredth meridian, to extreme northeastern Mexico (*2*). In areas in which the model is abundant, the dark morph of *P. glaucus* comprises a very high proportion of the female population, but apparently it nowhere totally replaces the light morph (Table 1). By contrast, where the range of *P. glaucus* exceeds that of *B. philenor*, the dark morph becomes scarce or disappears altogether, leaving a monomorphic light population. Thus, the dark morph is essentially absent north of southern New England, central New York, southern Michigan, southern Wisconsin, and southern Minnesota; it is uncommon in peninsular Florida, where the model is rare (*3, 4*). This decline in frequency of the dark morph both northward and southward indicates that it is not an adaptation to a gradient in an abiotic factor such as temperature or humidity (*3*).

The dimorphism apparently is under simple genetic control. Although the dark morph was earlier thought to be inherited as an autosomal dominant, sex-limited in expression (*5*), recent evidence shows that it is Y-linked (the female is the heterogametic sex) or, possibly, cytoplasmically transmitted. It follows that the dimorphism cannot be maintained by heterozygous advantage (*6*).

The geographically varying relative frequencies of the two morphs must reflect antagonistic selective forces. Pressure favoring the dark mimetic morph is exerted through differential predation on adults by birds that have experienced the model. One factor that might select against the dark morph is preferential mating of the monomorphic males with light females. Courtship studies in a number of butterfly species have demonstrated the importance of visual stimuli and the existence of male preference for females with malelike color-patterns (*7*). Although there is little direct evidence that females discriminate among males visually, departures from the primitive color-pattern by males are assumed to be far less acceptable to females than conservative dress because mimetic polymorphism in butterflies is frequently restricted to the female sex (*8*). Such restriction characterizes species emphasizing visual communication rather than those relying on olfactory stimuli, in which both sexes readily become mimetic (*9*). Male preference for primitive color-pattern is suggested for *P. glaucus* by courtship experiments in which light and dark females were tethered outdoors in Utah and Colorado, areas in which *P. glaucus* is replaced by three closely related western species of tiger swallowtails similar in appearance but not dimorphic. Males of two of these western swallowtails, *P. multicaudata* Kirby and *P. rutulus* Lucas, were far more attentive to the light morph of *P. glaucus* than to the dark morph (*10*).

To test for the existence of male mating preference in *P. glaucus*, samples of females were taken from two natural populations (Mt. Lake, Virginia, and Baltimore, Maryland) in which females are dimorphic (*11*); and, since male lepidopterans deposit sperm in membranous sac-like spermatophores rather than in amorphous ejaculates, the bursa copulatrix of each female was dissected and the spermatophores within were counted. For comparison, mating frequency was similarly investigated in *B. philenor* (*12*).

Two assumptions are made in determining mating frequency by counting the number of spermatophores in the bursa copulatrix: (i) the male transfers but one spermatophore per mating, and, (ii) even though the spermatophore commonly empties, collapses, and shrinks with time, its walls are persistent enough to be recognized. Studies on spermatophores and mating frequencies in several species of butterflies and skippers (*13*) and in a gelechiid moth (*14*) support these assumptions, although in some Lepidoptera more than one spermatophore may be introduced during a single mating and the spermatophore may be digested (*15*). However, with respect to the problem of preferential mating in *P. glaucus*, the

Table 1. Frequency, sample size, and date of capture of dark and light female morphs of *Papilio glaucus* from five localities in the southeastern United States.

Date of capture	No. of specimens	Frequency	
		Dark	Light
Baltimore, Maryland			
24 to 31 Aug. 1965	29	.45	.55
Mt. Lake, Va./W. Va.			
13 to 22 June 1965 26 July to 19 Aug. 1965	84	.86	.14
*Great Smoky Mountains North Carolina/Tennessee**			
11 to 21 Aug. 1954 22 July 1959	15	.93	.07
*Northern Georgia**			
15, 16 Aug. 1959	33	.97	.03
*Central Florida**			
6 Mar. to 13 June 1956	501	.06	.94
13 June to 6 Aug. 1959	517	.08	.92

* Data from Brower and Brower (*3*).