
ENZYMOLOGY:

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*J. Biol. Chem. 1966, 241:5910-5918.*

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 24, Issue of December 25, pp. 5910–5918, 1966
*Printed in U.S.A.*

# Mammalian Galactose Dehydrogenase

## II. PROPERTIES, SUBSTRATE SPECIFICITY, AND DEVELOPMENTAL CHANGES

(Received for publication, June 28, 1966)

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### SUMMARY

**The properties of a 100-fold purified preparation of rat liver galactose dehydrogenase are presented. Optimum activity occurs at pH 8.3. The enzyme is relatively stable to dialysis, heat treatment, and storage at −30°, but is quite unstable below pH 5. Nicotinamide adenine dinucleotide, but not galactose, protects from heat inactivation. Heat inactivation kinetics suggests that one enzyme is responsible for galactose oxidation.**

**Activity is strongly inhibited by sulfhydryl group inhibitors, $Cu^{++}$, $Zn^{++}$, and $Fe^{++}$, yet sulfhydryl reagents do not enhance activity or protect from inactivation. Activity was not inhibited by cyanide, arsenate, or ethylenediaminetetraacetate.**

**The $K_m$ for galactose is $2.6 \times 10^{-2}$ M and for NAD is $1.7 \times 10^{-4}$ M. The $V_{max}$ is 0.57 μmole per min per mg of protein. The reaction product, NADH, is a strong competitive inhibitor of galactose dehydrogenase ($K_i$, $3.4 \times 10^{-5}$ M). The cofactor NADP is less than 1% as effective as NAD, and it competitively inhibits ($K_i$, $6.6 \times 10^{-3}$ M). NADPH is also a competitive inhibitor ($K_i$, $1.2 \times 10^{-3}$ M).**

**The enzyme preparation has a very broad specificity for sugar substrates, reduction of NAD (but not of NADP) occurring in the presence of various aldohexoses, aldopentoses, aldoheptoses, and disaccharides. The specificity patterns suggest the presence of at least three aldose dehydrogenases with very specific steric requirements for substrates. Studies with combinations of sugars, heat inactivation, and starch gel electrophoresis were more consistent with the presence of a single enzyme of unusual substrate specificity. Heat inactivation kinetics suggests two maltose-oxidizing enzymes. The NAD-reduction reactions occurring with L-aldohexoses, aldoheptoses, maltose, and cellobiose suggest novel reactions hitherto unrecognized in mammalian tissues. Some keto sugars behave as substrates, probably by isomerization to corresponding aldo sugars, but an unusual direct oxidation was not excluded.**

**Developmental changes in rat liver galactose dehydrogenase show that activity is barely detectable until birth, when a sudden increase occurs, reaching a maximum in animals 5 days old and falling slowly to the adult level in animals 30 days of age.**

The detection and partial purification of a soluble mammalian liver nicotinamide adenine dinucleotide-dependent galactose dehydrogenase (D-galactose:NAD oxidoreductase, EC 1.1.1.48) has been described (1). It catalyzes the following reaction.

D-Galactose + $NAD^+$ ⇄ D-galactonolactone + NADH + $H^+$

This appears to be the initial step in the conversion of D-galactose to D-xylulose by a series of reactions which could possibly serve as an alternative oxidative pathway for galactose in mammalian tissues (2).

The existence in bacterial systems of various carbohydrate oxidase reactions resulting in corresponding aldonic acids is well established. Some of these exhibit broad substrate specificity (3–6). In mammalian tissues, the study of sugar dehydrogenase reactions has been largely limited to those involving oxidation of glucose by liver (7–12) and of D-xylose and D-glucose by lens (13, 14). This paper presents some data on the properties of rat liver galactose dehydrogenase. The presence in liver of some novel NAD-dependent sugar-oxidation reactions is suggested.

### EXPERIMENTAL PROCEDURE

*Materials*—The sodium salts of NADH and NADPH were obtained from Sigma and dissolved in 0.01 M Tris buffer, pH 8.1. EDTA was purchased from Fisher Scientific Company and solutions were adjusted to pH 8 with NaOH. *p*-Chloromercuribenzoate, dithiothreitol, *N*-ethylmaleimide, and iodoacetic acid were obtained from Calbiochem. NAD, NADP, Tris buffer, phenazine methosulfate, and mercaptoacetic acid were purchased from Sigma. Nitro blue tetrazolium was obtained from Nutritional Biochemicals. Hydrolyzed starch for gel electrophoresis was obtained from Connaught Medical Research Laboratories. Crystalline potassium D-galactonate was prepared from glucose-free galactose by the hypoiodite oxidation method of Moore and Link (15). All other sugars were commercially obtained except for the following, which were provided by Dr. Nelson K. Richtmyer, National Institutes of Health: L-rhamnose, D-fucose, D-gulose, D-allose, L-allose, D-altrose, D-sorbose, L-galactose, D-gluco-D-guloheptose, D-manno-D-galactoheptose, D-manno-heptulose, D-altroheptulose, and D-glucoheptulose. All the sugars which served as substrates were checked for homogeneity by paper chromatography on ethyl acetate-pyridine-water, 12:5:4. All other reagents were of analytical grade.

*Analytical Procedures*—All studies unless otherwise indicated

were performed with a partially purified (100-fold) Sprague-Dawley rat liver galactose dehydrogenase (2.2 mg per ml) described in the preceding paper (1), and assay methods were also as previously described. Galactose dehydrogenase activity was assayed at room temperature by following the rate of NAD reduction at 340 mμ, and assays contained 100 μmoles of galactose, 3 μmoles of NAD, 100 μmoles of Tris buffer, and appropriate aliquots of enzyme (5 to 10 μl) in a final volume of 1.0 ml, pH 8.1. In all cases, the enzyme was added to initiate the reaction. A unit of activity was defined as the amount of enzyme required to reduce 1 mμmole of NAD per min. Specific activity was expressed as units per mg of protein. Protein determinations were performed by the method of Lowry *et al.* (16).

The aldonic acid derivatives resulting from the oxidation of various sugars which served as substrates with the enzyme preparation were converted to the corresponding lactones by heating in 1 N HCl for 5 min. The lactones were measured colorimetrically as the hydroxamate derivatives by the method of Hestrin (17). In these experiments, a mixture containing 50 μmoles of sugar substrate, 3 μmoles of NAD, 50 μmoles of Tris buffer, 0.1 μmole of menadione, 90 μg of NADH oxidase, and 120 μg of enzyme in a total volume of 0.5 ml, pH 8.1, was incubated for 5 hours at 30°. Aldonic acids were determined at zero time and at 5 hours.

In heat inactivation studies, the enzyme (0.3 to 0.5 ml) was placed in a water bath of the desired temperature and aliquots (40 μl) were transferred at given times to microcentrifuge tubes kept at 4°. The denatured protein was removed by centrifugation for 5 min at 10,000 × *g* and 5 μl of the supernatant were used in the enzyme assay.

Starch gel electrophoresis was performed with a vertical apparatus. Concentration of hydrolyzed starch was 13.5 g/100 ml of 5.3 mM phosphate buffer; final gel pH was 6.6. Electrode buffer was 0.02 M phosphate, pH 6.7. Running time was about 15 hours at 200 volts, and the temperature was about 8°. Following each run, the gels were sliced and stained by incubating at room temperature for about 45 min in the following solution: phenazine methosulfate, 20 μg per ml; NAD, 1 mM; nitro blue tetrazolium, 0.5 mg per ml; sodium cyanide, 1 mM; Tris buffer (pH 8.4), 50 mM; and the substrate sugar, 50 mM.

In the studies of developmental changes of enzyme activity, the assay system was as described above except that crude enzyme preparations were used. Liver was homogenized with 4 volumes of 0.01 M phosphate buffer, pH 6.9, and centrifuged at 32,000 × *g* for 1 hour. The supernatant was the enzyme source used. Young Sprague-Dawley rats were kept with their mother until 40 days of age. Gestational age of pregnant animals was estimated from the date of mating.

## RESULTS

*Enzyme Proportionality*—The amount of NAD reduced by galactose dehydrogenase was proportional to the length of incubation over a wide range of protein concentrations (Fig. 1). Loss of linearity occurred with protein concentrations greater than 25 μg per ml, or with lower concentrations but longer incubation periods. This was always related to the final concentration of NADH achieved, and was consistent with the studies of NADH inhibition discussed below. Accurate initial velocities were obtained since in all subsequent studies the protein concentration was 10 to 20 μg per ml, and incubations were for 6 to 10 min.

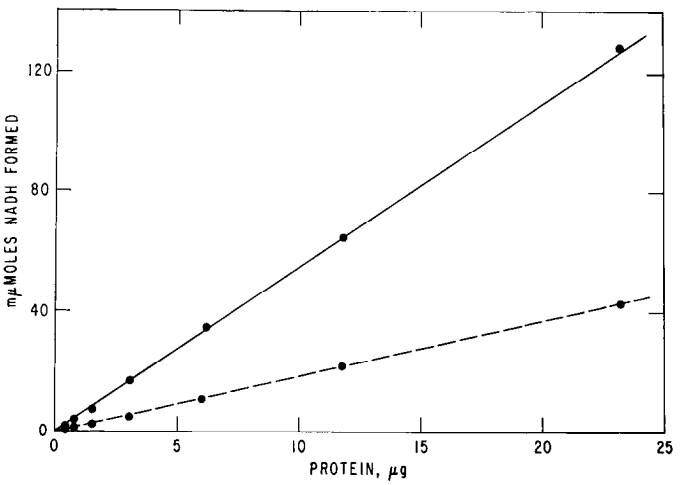


FIG. 1. Proportionality of galactose dehydrogenase activity to protein concentration. Assay was as described in text. Incubations were for 6 min (- - -) or 18 min (——) at room temperature.

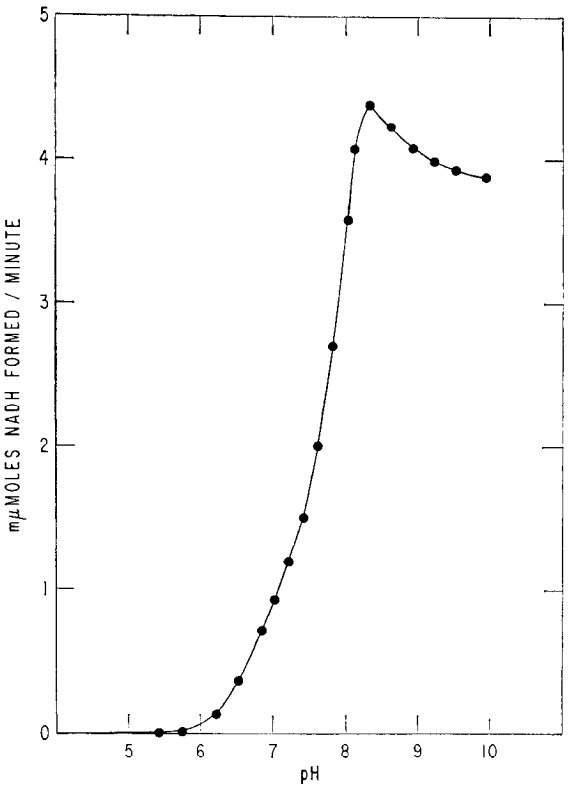


FIG. 2. Effect of pH on galactose dehydrogenase activity. Phosphate buffer was used for pH range, 5.4 to 7.8, Tris-HCl buffer for pH 7.6 to 8.8, and glycine buffer for pH 8.6 to 9.9. Eleven micrograms of enzyme were used in the incubation mixture.

*Effect of pH on Enzyme Activity*—The activity of galactose dehydrogenase was tested over the pH range, 5.4 to 9.9 (Fig. 2). Optimum activity occurred at pH 8.3. A similar curve was obtained if the galactose concentration was decreased from 0.1 M to 0.025 M. A human liver galactose dehydrogenase preparation, purified 5-fold, had a pH optimum of 8.6.

*Enzyme Stability*—Dialysis for 16 hours at 5° with 500 volumes

of 0.01 M phosphate buffer, pH 6.9, resulted in 9% loss of activity. Addition of 2 mM EDTA to the dialysis medium did not cause further loss of activity. Galactose dehydrogenase was very unstable in acid pH, total and irreversible inactivation occurring rapidly below pH 5.4. The enzyme was quite stable at pH 6.3 to 9.1. The frozen (−30°) purified enzyme retained 70% and 63% of its activity after storage for 4 and 6 months, respectively; when frozen in the presence of 1 mM NAD, the respective activities were 86% and 78% at 4 and 6 months. Potassium chloride (50 mM), NaF (8 mM), and dithiothreitol (1 mM) did not enhance stability to storage.

Galactose dehydrogenase is relatively heat stable, little loss in activity occurring after heating for 15 min at 45°. NAD afforded considerable protection from heat inactivation (Fig. 3). After 20 min at 55°, the enzyme had lost 55% of its activity in the absence of NAD compared to 26% in the presence of 1 mM NAD. No such protection was seen with 5 or 50 mM galactose, and galactose and NAD together had no more stabilizing effect than NAD alone. Furthermore, the inactivation curves display kinetics of a first order reaction, suggesting that a single enzyme is responsible for the activity being measured.

*Inhibitors*—*p*-Chloromercuribenzoate and *N*-ethylmaleimide strongly inhibited galactose dehydrogenase activity (Table I). The effects were immediate since initial velocities were fully depressed 2 min after exposure of the enzyme to the reagent. Iodoacetic acid also caused a fall in activity, but much higher concentrations were required. Dithiothreitol afforded some protection from *p*-chloromercuribenzoate inactivation at 0.5 mM, but higher concentrations were not more effective. If the enzyme was preincubated with 0.1 mM *p*-chloromercuribenzoate for 20 min at 20°, no activity returned on adding dithiothreitol. These findings suggested that sulfhydryl groups of the enzyme are necessary for activity. However, the sulfhydryl compounds, dithiothreitol, mercaptoacetic acid, and cysteine at 1 mM, did not enhance enzyme activity, and they did not provide protection from heat inactivation. There was no inhibition by EDTA or cyanide, suggesting that metallic ion cofactors are not involved in enzyme activity. The divalent cations, $Cu^{++}$, $Zn^{++}$, and $Fe^{++}$, caused inhibition of activity (Table I).

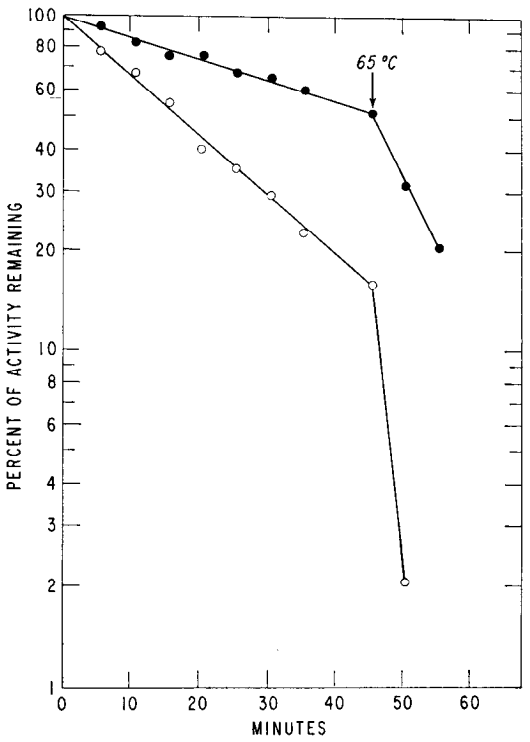


FIG. 3. Heat inactivation of galactose dehydrogenase activity at 55° in the absence (○——○) and in the presence of 1 mM NAD (●——●). Temperature was increased to 65° at 45 min (*arrow*). The enzyme was heated in 0.01 mM phosphate buffer and the pH was 6.8. Procedure for assay was as described in text.

TABLE I

*Effect of various reagents on galactose dehydrogenase activity*

Enzyme assay was as described in text. The enzyme (22 μg) was added to the incubation mixture containing the reagent being studied, and absorbance readings were obtained every minute for 10 min.

| Reagent | Concentration | Control activity |
|---|---|---|
| | mM | % |
| *p*-Chloromercuribenzoate | 0.01 | 72.5 |
| *p*-Chloromercuribenzoate | 0.1 | 0 |
| Plus dithiothreitol | 0.1 | 0 |
| Plus dithiothreitol | 0.5 | 22.3 |
| Dithiothreitol | 0.5 | 100 |
| *N*-Ethylmaleimide | 0.1 | 19.4 |
| Iodoacetic acid | 0.1 | 100 |
| Iodoacetic acid | 1.0 | 40.1 |
| Cysteine | 1.0 | 100 |
| Mercaptoacetic acid | 1.0 | 100 |
| EDTA | 5.0 | 100 |
| NaCN | 1.0 | 100 |
| NaF | 8.0 | 100 |
| Arsenate | 5.0 | 100 |
| $CuSO_4$ | 0.5 | 0 |
| $ZnSO_4$ | 0.5 | 20.1 |
| $FeCl_2$ | 0.3 | 63.5 |
| $MnCl_2$ | 0.3 | 100 |
| $MgCl_2$ | 0.3 | 100 |
| $NH_4SO_4$ | 0.5 | 100 |
| $NH_4SO_4$ | 5.0 | 100 |

*Maximal Velocities and Michaelis Constants*—The Michaelis constant ($K_m$) for galactose from Lineweaver-Burk plots at saturating concentrations of NAD (3 mM) was $2.6 \times 10^{-2}$ M. The $K_m$ for NAD (galactose, 0.1 M) was $1.7 \times 10^{-4}$ M. The $V_{max}$ for galactose (NAD, 3 mM) was 0.57 μmole per min per mg of protein.

NADH was a strong inhibitor of galactose dehydrogenase activity. It acted as a noncompetitive inhibitor of galactose (Fig. 4) when a subsaturating concentration of NAD (1 mM) was used. Competition was clearly competitive (Fig. 5*A*) when the NAD concentration was varied (galactose, 0.1 M). These kinetic data, when analyzed according to Cleland's formulations, show a product inhibition pattern consistent with an ordered (nonrandom) reaction (18).[1] Furthermore, the data suggest that the simplest mechanism for the dehydrogenase reaction is an ordered "Bi Bi" mechanism, although a Theorell-Chance mechanism cannot be excluded. The first substrate to bind to the enzyme would be NAD and the second galactose, and the first product to come off the enzyme would be galactonolactone, followed by NADH. The $K_i$ for NADH, calculated

[1] We are indebted to Dr. John Hearon and Dr. Wayne London for guidance in the analysis of the kinetic data.

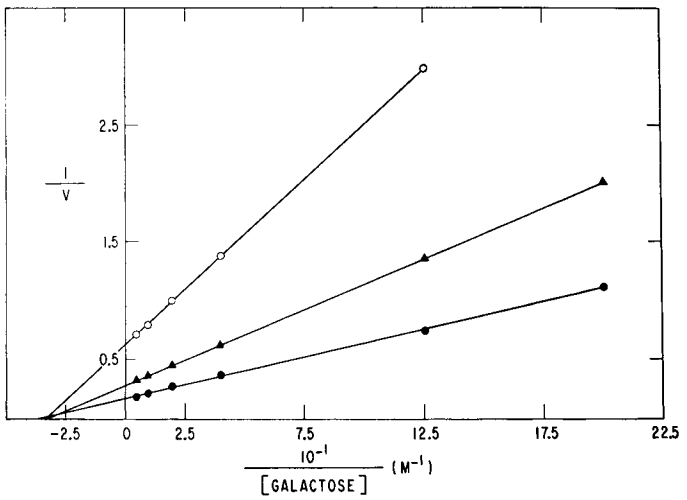


FIG. 4. Effects of NADH on plots of reciprocal initial velocity with respect to reciprocal galactose concentration for galactose dehydrogenase system. Incubations were as described in text (●——●), except for the additional presence of 0.12 mM (▲——▲) and 0.38 mM (○——○) NADH in those two plots. NAD concentration was 1 mM. Initial velocity is expressed as 10 times the amount of NAD reduced (micromoles) per min per mg of protein.

according to an ordered Bi Bi (or Theorell-Chance) mechanism, is $3.4 \times 10^{-5}$ M.

NADP was less than 1% as effective as NAD as a cofactor in the galactose dehydrogenase reaction. It was a competitive inhibitor for NAD (Fig. 5*B*), and had a $K_i$ of $6.6 \times 10^{-3}$ M. NADPH was a somewhat more potent competitive inhibitor and had a $K_i$ of $1.2 \times 10^{-3}$ M (Fig. 5*B*).

When enzyme activity was studied at pH 7.4, no inhibition of activity was detected with the reaction product, D-galactonolactone.

*Substrate Specificity*—Table II compares the initial velocities of galactose dehydrogenase obtained with a variety of aldohexoses. The steric configurations of these sugars suggest that two groups of sugars (D- and L-aldohexoses) with distinct conformational requirements can serve as substrates.[2] These two groups are represented in Fig. 6 by D-galactose and L-allose. All D-aldohexoses which serve as substrates possess an axial hydroxyl group at C-4, and no sugar with such an orientation is inactive. C-6 appears necessary for activity, since L-arabinose was not a substrate and loss of the C-6 hydroxyl group results in decreased activity (D-fucose). The presence of a C-6 carboxyl group results in total loss of activity (D-galacturonic acid). The C-2 hydroxyl group is unnecessary since 2-deoxygalactose has 100% activity, and additions in this position (D-galactosamine, *N*-acetyl-D-galactosamine) apparently do not hinder favorable binding of the substrate to the enzyme. However, changing the C-2 hydroxyl group to an axial orientation results in enhanced activity (D-talose). The C-3 hydroxyl group is preferred in an equatorial orientation since D-galactose is a better substrate than D-gulose. The L-aldohexoses which serve as substrates have the C-4 hydroxyl group in an equatorial orientation (Fig. 6), and those which are not substrates have an axial orientation at this carbon atom (with the exception of L-glucose). Substitution of NADP for NAD results in a marked fall or total absence of activity for all aldohexoses tested (Table II).

[2] We are indebted to Dr. Frank Eisenberg for guidance in the formulations of configurational specificity.

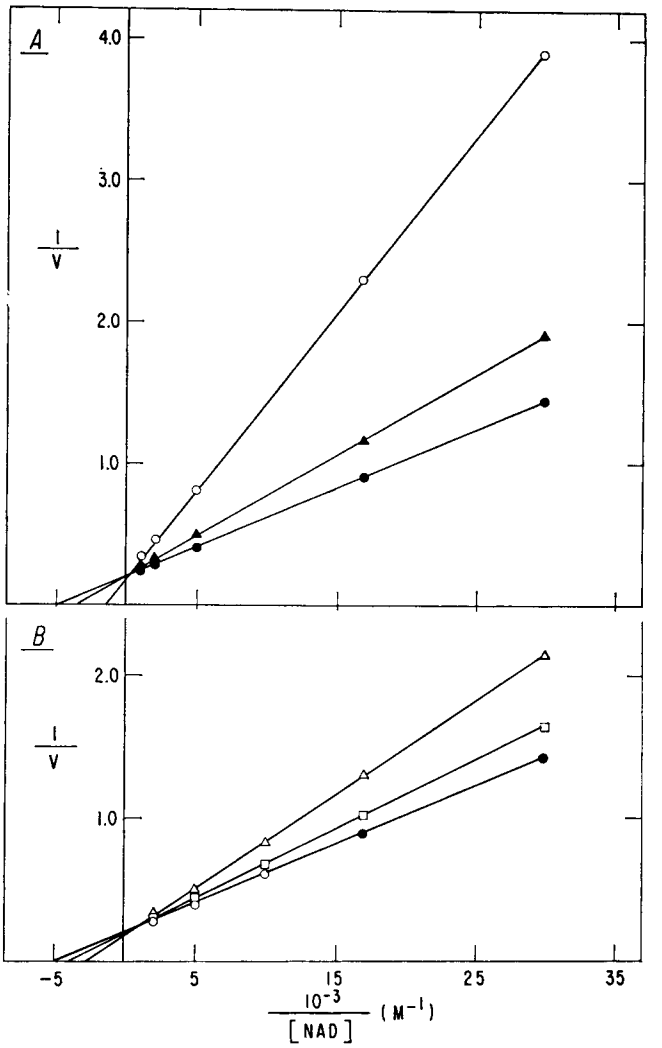


FIG. 5. Lineweaver-Burk analysis of the effects of NADH (7.5 μM, ▲——▲; 75 μM, ○——○), NADPH (0.22 mM, □——□), and NADP (4.0 mM, △——△) on galactose dehydrogenase activity. Incubations were as described in text (●——●). Galactose concentration was 0.1 M. Initial velocity is expressed as in Fig. 4.

TABLE II

*Aldohexose specificity of dehydrogenase*

The assay procedure is as described in text. All sugars were tested at a concentration of 50 mM. NAD and NADP were 3 mM.

| Aldohexose | Relative rate | | Aldohexose | Relative rate | |
|---|---|---|---|---|---|
| | NAD | NADP | | NAD | NADP |
| D-Galactose | 100 | 1 | D-Fucose | 3 | 0 |
| L-Galactose | 0 | 0 | L-Fucose | 0 | 0 |
| D-Mannose | 0 | 0 | L-Rhamnose | 55 | 2 |
| L-Mannose | 32 | 0 | D-Rhamnose | 0 | 0 |
| D-Glucose | 0 | 0 | *N*-Acetylgalactosamine | 108 | 2 |
| L-Glucose | 0 | 0 | D-Galactosamine | 106 | 1 |
| D-Allose | 0 | | 2-Deoxy-D-galactose | 100 | 2 |
| L-Allose | 105 | 1 | D-Galacturonic acid | 0 | 0 |
| D-Altrose | 0 | | D-Galactonic acid | 0 | 0 |
| D-Gulose | 48 | 0 | D-Galactono-γ-lactone[a] | 0 | 0 |
| D-Talose | 120 | 3 | | | |

[a] Tested at pH 7.3.

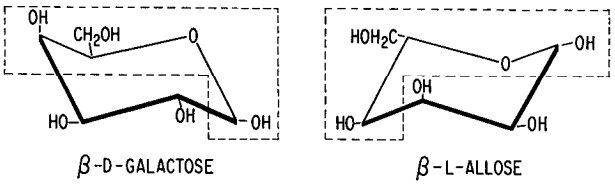


FIG. 6. Proposed conformational specificity of substrates of postulated D- and L-aldohexose dehydrogenases. The configuration indicated by the *dashed lines* is necessary for detectable oxidation of the sugar.

TABLE III

*Action of other sugars as substrates for galactose dehydrogenase enzyme preparation*

Assay procedure is as described in text, with concentrations of 50 mM for sugars and 3 mM for NAD. Relative rate is based on D-galactose being 100.

| Substrate | Relative rate | Substrate | Relative rate |
|---|---|---|---|
| Pentoses | | | |
| D-Arabinose[a] | 112 | Keto sugars | |
| L-Arabinose | 0 | D-Sorbose | 215 |
| D-Ribose[b] | 10 | D-Tagatose | 133 |
| D-Xylose[b] | 84 | L-Sorbose[b] | 2 |
| L-Xylose[a] | 155 | D-Fructose[b] | 5 |
| D-Lyxose[a] | 22 | D-Mannoheptulose | 110 |
| Aldoheptoses | | D-Altroheptulose | 0 |
| D-Gluco-D-guloheptose | 0 | D-Glucoheptulose | 27 |
| D-Manno-D-galactoheptose | 100 | Sugar alcohols | |
| Disaccharides | | D-Dulcitol[b] | 0 |
| Lactose | 0 | D-Sorbitol[b] | 0 |
| Maltose | 163 | | |
| Sucrose | 0 | | |
| Melibiose | 0 | | |
| Cellobiose | 161 | | |

[a] Activity is present with 3 mM NADP but the rate is less than 3%.

[b] Activity not present with NADP. Other sugars were not tested with NADP.

A variety of other sugars was active in the NAD-reduction reaction with the partially purified enzyme preparation (Table III). The pentoses which served as substrates do not conform to the aldohexose specificity patterns. However, the D-pentoses may form a separate group of oxidizable sugars which have in common the presence of the C-4 hydroxyl group in an axial orientation. L-Xylose sterically resembles the active D-aldohexose sugars. Two of the disaccharides tested, maltose and cellobiose, were good substrates, but it is difficult to define a specificity pattern.

The activity present with the keto sugars can possibly be explained by an isomerization reaction resulting in interconversion of the keto to the aldo sugar. If such were the case, the specificity pattern would fit that described for the aldohexoses. To test for the presence of such an isomerase reaction, D-tagatose (0.1 M) was incubated with 200 μg of enzyme at 30° in a total volume of 2.5 ml, pH 8.2. Formation of the isomerization product, D-galactose or D-talose, was confirmed by assaying with galactose oxidase, which is active with either of these substrates but not with D-tagatose (19). It is not clear whether the rate of isomerization (0.16 μmole per hour per 2.5 ml) is sufficiently rapid to account for the high activity of oxidation shown for D-tagatose or D-sorbose. However, 1.32 μmoles of aldonic acid were also formed in the above experiment if NAD was added. This suggested that the product of isomerization was being oxidized, as expected if D-galactose or D-talose had been the product. The over-all reaction may represent an isomerase-dehydrogenase catalysis by a single enzyme. Such a reaction has been described for liver alcohol dehydrogenase, where in addition to dehydrogenation the enzyme can catalyze in the presence of NAD the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (20, 21).

The reaction products of sugars other than galactose have not yet been definitively identified. Aldonic acids were detected following incubation of some D- and L-aldohexoses, pentoses, and disaccharides with the enzyme preparation (Table IV). The two nonsubstrate sugars tested, L-arabinose and lactose, were not converted to aldonic acids. It seems reasonable that the aldose sugars are oxidized to the corresponding lactones.

It seemed on the basis of substrate specificity that the large number of sugars oxidized was in part due to the presence of more than one aldose dehydrogenase in the partially purified rat liver preparation. Unfortunately, the inability to obtain greater purification (1) hindered a thorough study of this question. Table V shows the results of experiments in which various substrate sugars at nearly saturating concentrations were combined with galactose at 0.1 and 0.2 M concentration. No additive effect of activities was found for D-arabinose, L-rhamnose, L-mannose, and D-xylose. This suggested the action of a single enzyme, but could also have resulted from inhibition of one of two hypothetical enzymes by one of the two substrates present in the reaction mixture. The results obtained when maltose was added to galactose also suggested a single enzyme activity, but these studies are complicated by the substrate inhibition which maltose exhibits.

Heat inactivation studies showed parallel loss of enzyme activity with D-galactose, D-arabinose, and L-rhamnose (Fig. 7), suggesting that the activity for these sugars was produced by the same enzyme or by different enzymes with similar heat

TABLE IV

*Production of aldonic acid by incubating various sugars with galactose dehydrogenase preparation*

The values represent the amount of aldonic acid produced in 5 hours in 0.5 ml of incubation mixture, as described in text.

| Substrate | Aldonic acid produced |
|---|---|
| | *μmoles* |
| D-Gulose | 0.11 |
| L-Mannose | 1.01 |
| L-Rhamnose | 0.56 |
| D-Arabinose | 0.41 |
| L-Arabinose | <0.07 |
| D-Xylose[a] | 0.19 |
| L-Xylose | 0.40 |
| Maltose | 0.40 |
| Cellobiose | 0.59 |
| Lactose | <0.07 |

[a] Complete recovery of xylonolactone would not be expected, since this would require longer heating than was performed here (13).

stability. Similar curves, not shown in Fig. 8, were obtained with L-mannose, D-gulose, and D-sorbose. The inactivation curve for maltose differed in having an initial fall which paralleled that seen with the other substrates, followed by stabilization at 20% of the original activity. This suggests that with maltose as substrate at least two enzymes are involved, one of which is more heat stable than the one oxidizing the other sugars. The other is indistinguishable from the galactose-utilizing enzyme, and probably accounts for most (80%) of the maltose activity measured.

Starch gel electrophoresis of the partially purified galactose dehydrogenase preparation confirmed the observation that on ion exchange resins it behaves like a basic protein (1), since migration was toward the cathode at pH 6.5 to 7.8. With a staining technique specific for enzyme activity, no major differences in electrophoretic mobility were detected when various sugars were used as substrates.

Table VI shows the $K_m$ and $V_{max}$ values of 11 sugars which had demonstrable activity with the partially purified enzyme preparation. All $K_m$ values are relatively large, but that for D-lyxose is so high that it is difficult to suggest physiological significance. The smallest $K_m$ values were obtained with the disaccharides, maltose and cellobiose. Lineweaver-Burk plots for four of the sugars listed in Table IV are shown in Fig. 8. Cellobiose and maltose exhibit substrate inhibition.

Some of the sugars which were not substrates were inhibitors of enzyme activity when galactose served as the substrate (Fig. 9). The three hexoses, D-glucose ($K_i$, 0.085 M), D-fucose ($K_i$, 0.075 M), and D-mannose ($K_i$, 0.054 M), were competitive inhibitors, whereas the pentose, L-arabinose ($K_i$, 0.180 M), was a noncompetitive inhibitor. Fructose, a weak substrate, did not inhibit galactose activity at 0.1 or 0.2 M.

*Developmental Changes in Liver Galactose Dehydrogenase Activity*—The galactose dehydrogenase activity of embryonic rat liver was extremely low (Fig. 10). Enzyme activity was undetectable until shortly before birth (20-day fetus), but increased sharply at birth and continued to rise to reach a maximum in animals 5 days old. There was a 10-fold increase in specific activity (1.6 to 15.6) during this 6-day period. After 5 days, the activity decreased slowly to reach the adult level of 6.7 in

TABLE V

*Activity of dehydrogenase obtained by combining galactose with various other substrate sugars*

Assay is as described in text. Activity values given are initial velocities expressed as 100 times the change in absorbance at 340 mμ per min per 10 μl of enzyme preparation.

| Added sugar | | Activity | | |
|---|---|---|---|---|
| Type | Concentration | No galactose | 0.1 M galactose | 0.2 M galactose |
| | M | | | |
| None | | | 26.3 | 32.1 |
| D-Arabinose | 0.1 | 33.2 | 38.6 | |
| | 0.2 | 37.5 | | 36.9 |
| L-Rhamnose | 0.1 | 16.1 | 25.2 | |
| | 0.2 | 20.4 | | 32.8 |
| L-Mannose | 0.1 | 8.3 | 24.2 | |
| | 0.2 | 12.0 | | 29.1 |
| D-Xylose | 0.1 | 22.6 | 27.8 | |
| | 0.2 | 26.5 | | 36.1 |
| Maltose | 0.1 | 21.3 | 20.3 | |
| | 0.2 | 13.0 | | 12.8 |

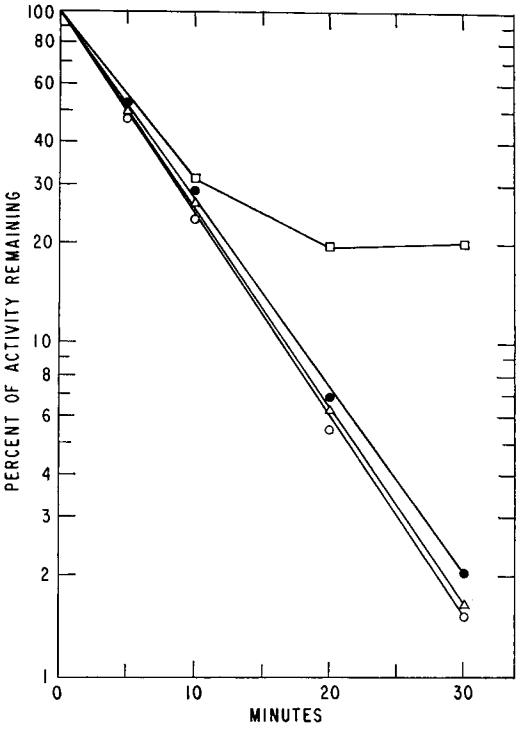


FIG. 7. Heat inactivation of dehydrogenase enzyme activity at 60° measured with 50 mM D-galactose (●——●), D-arabinose (△——△), L-rhamnose (○——○), or maltose (□——□) as the substrate in the assay as described in text. The enzyme was heated in 0.1 M phosphate buffer and the pH was 6.8.

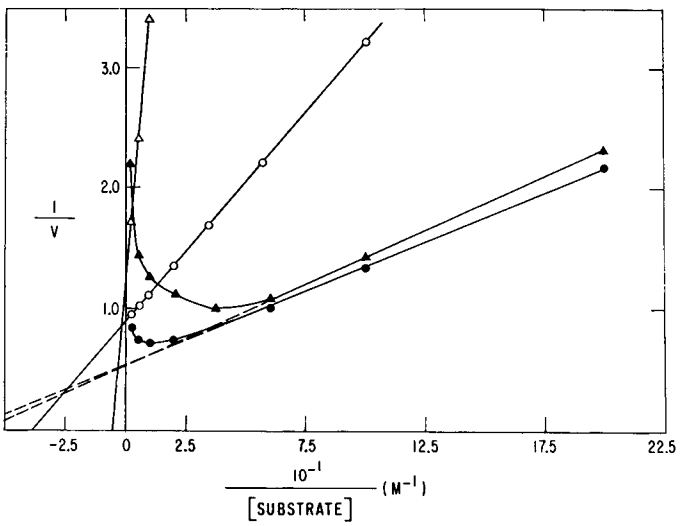


FIG. 8. Plot of reciprocal initial velocity with respect to reciprocal concentration of D-galactose (○——○), cellobiose (▲——▲), maltose (●——●), and L-mannose (△——△). Initial velocity is expressed as in Fig. 4, and incubation procedures were as described in text.

animals 30 days old. The galactose $K_m$ and $V_{max}$ obtained with newborn rat liver preparations were the same as those obtained with the adult liver preparation, and the $K_m$ was very similar to that obtained with the purified liver enzyme. There was no difference in the liver galactose dehydrogenase specific activity of male, female, and lactating rats.

*Adaptive Aspects of Liver Galactose Dehydrogenase Activity*—There was no change in liver galactose dehydrogenase specific activity in male or female adult rats after 7 days of feeding *ad libitum* on a 40% galactose diet. Rats 15 days pregnant were given a 40% galactose diet and killed at 17 and 18 days to determine whether dietary galactose given to the mother could induce higher enzyme activities in the fetal livers. No effects were seen since all fetal livers tested had no detectable enzyme activity.

TABLE VI

*Michaelis-Menten constants of dehydrogenase reaction for various sugars*

The values were obtained by the method of Lineweaver and Burk (22). The assay was as described in text. For $K_m$ and $V_{max}$ studies, the sugar concentrations ranged from 1 mM to 400 mM. NAD concentration was 1 mM.

| Compound | $K_m$ | $V_{max}$ |
|---|---|---|
| | | *μmole/min/mg* |
| D-Lyxose | 0.4 | 0.23 |
| L-Mannose | 0.18 | 0.23 |
| D-Ribose | 0.16 | 0.27 |
| L-Rhamnose | 0.062 | 0.54 |
| D-Xylose | 0.040 | 0.61 |
| D-Galactose | 0.026 | 0.57 |
| 2-Deoxy-D-galactose | 0.026 | 0.57 |
| L-Xylose | 0.023 | 0.75 |
| D-Arabinose | 0.019 | 0.82 |
| Cellobiose | 0.016 | 0.91 |
| Maltose | 0.015 | 0.100 |

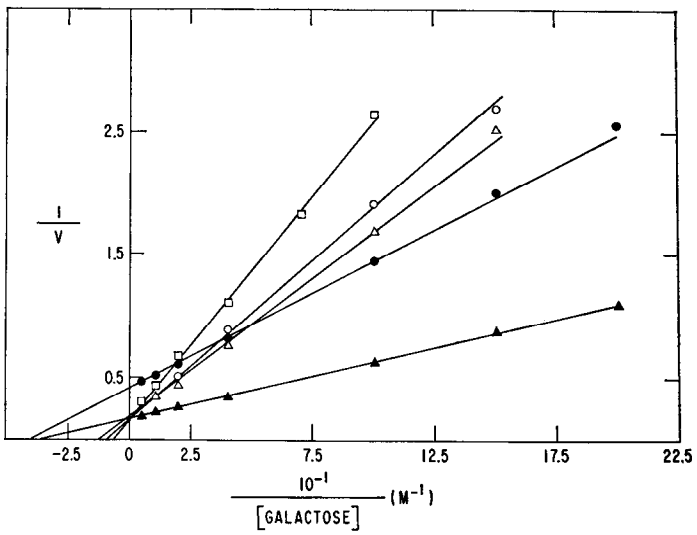


FIG. 9. Lineweaver-Burk analysis of the effects of various non-substrate sugars on galactose dehydrogenase activity. Incubation was as described in text for galactose (▲——▲), except for the additional presence of 0.2 M L-arabinose (●——●), D-glucose (△——△), D-fucose (○——○), and D-mannose (□——□). Initial velocities are expressed as in Fig. 4.

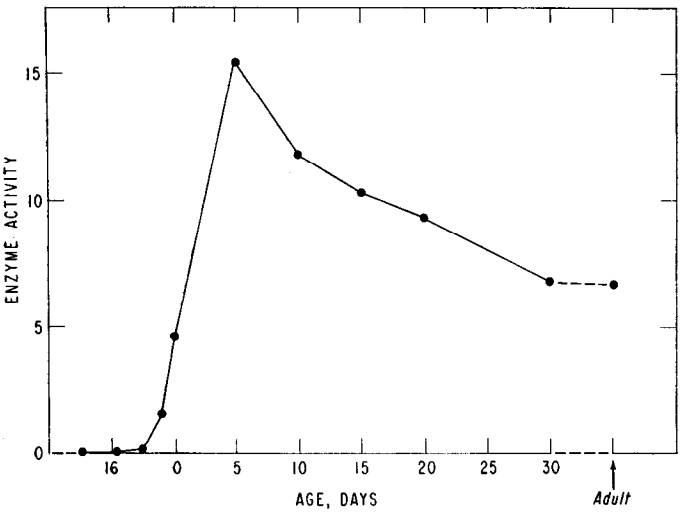


FIG. 10. Changes in galactose dehydrogenase activity in the developing rat liver. All animals older than newborn were male. Each *point* represents the average of two to four animals. Livers from an entire litter were pooled for the 14-, 16-, and 18-day fetus determinations, two separate litters being used in each case. The fetal livers from the same litter were pooled for the 20-day value; two litters were used. Fetuses are represented by gestational age. Enzyme activity is expressed as specific activity.

*Some Properties of Human Liver Galactose Dehydrogenase*—A galactose dehydrogenase extract of human liver, obtained *post mortem* from a 40-year-old white male, was purified 5-fold by 100,000 × *g* centrifugation, heat treatment, and ammonium sulfate as described previously (1). It was dialyzed for 24 hours against 500 times its volume of 0.01 M phosphate buffer, pH 7.0. As in the rat, human liver galactose dehydrogenase is found in the soluble cellular fraction. The preparation was found to have a pH optimum of 8.6. It was inactivated by heat (55°) linearly in a manner similar to that of rat liver galactose dehydrogenase (Fig. 3), but was considerably more heat stable. In the presence of 1 mM NAD, there was almost complete protection against heat inactivation at 55°. The Michaelis constant ($K_m$) for galactose at 3 mM NAD was $2.4 \times 10^{-2}$ M, and for NAD at 0.1 M galactose the $K_m$ was $2.8 \times 10^{-4}$ M. The $V_{max}$ for galactose (NAD, 3 mM) was 8.0 mμmoles per min per mg of protein with the crude preparation.

## DISCUSSION

There are very few examples of mammalian dehydrogenase reactions in which nonphosphorylated aldo sugars are oxidized. The presence in mammalian liver of a dehydrogenase which catalyzes the oxidation of glucose to gluconolactone is well established (7–12). Unlike galactose dehydrogenase, it is almost exclusively a particulate enzyme (10). Glucose dehydrogenase also differs from galactose dehydrogenase in that it is insensitive to sulfhydryl group inhibitors (10, 12), is able to utilize either NAD or NADP (10, 12, 23), and has a larger $K_m$ for its sugar substrate, varying, in rat liver, from 0.3 to 0.7 M (10). Glucose dehydrogenase can use both D-glucose and D-xylose as substrates. With a relatively crude lamb liver extract, Wainio observed oxidation of D-glucose, D-arabinose, and D-xylose in the presence of methylene blue when NAD and NADP were added to the system (24). van Heyningen has shown NAD-dependent con-

version of glucose and xylose to gluconic and xylonic acids, respectively, by calf lens extracts (13, 14).

The wide spectrum of substrates which were active with the present rat liver dehydrogenase preparation indicates that the direct oxidation of sugars by mammalian tissues may be a more general phenomenon than heretofore suspected. Some bacterial dehydrogenase systems of broad substrate specificity have been described. *Pseudomonas fragi* contains a particulate aldose dehydrogenase which oxidizes two hexoses (D-galactose and D-glucose) as well as three pentoses (L-arabinose, D-xylose, D-ribose), but which could not use NAD or NADP as hydrogen acceptors (3). Recently, Cline and Hu have isolated three sugar dehydrogenases from *Pseudomonas saccharophila* (5, 25–27): a heat-labile NAD-dependent aldose dehydrogenase active with D-glucose, D-galactose, and L-arabinose, an NADP-dependent galactose dehydrogenase active with galactose, L-arabinose, and D-fucose, and an NAD-dependent D-arabinose dehydrogenase. The liver dehydrogenase herein described does not conform to any of these patterns, and differs from the more nonspecific bacterial aldose dehydrogenases in being unable to oxidize both glucose and galactose.

The substrate specificity patterns shown by the rat liver enzyme preparation suggest that mammalian liver may contain aldose dehydrogenases specific for D-hexoses, L-hexoses, D-pentoses, and perhaps heptoses and disaccharides. Attempts to separate these enzymes have so far failed, with the possible exception of the maltose reaction which shows a biphasic rate of heat inactivation characteristic of two separate first order reactions. Although unlikely, the possibility remains of a single enzyme with peculiarly broad specificity. The precise elucidation of this problem must wait until further purification of the enzyme is achieved.

Precedents exist in some nonmammalian species of relatively broad specificity sugar oxidase systems. One of these is the carbohydrate oxidase of the marine red alga, *Iridophycus flaccidum*, which oxidizes D-galactose and D-glucose to the corresponding aldonic acids, and the disaccharides maltose, lactose, and cellobiose to the corresponding aldobionic acids (28). An enzyme has been isolated from the juice sacs of young oranges which oxidizes cellobiose, maltose, lactose, D-glucose, D-mannose, D-xylose, and D-galactose to the corresponding aldonic acids (29). Also, a D-fructose dehydrogenase has recently been isolated from a *Gluconobacter cerinus* which is active in the oxidation of D-fructose, D-galactose, D-glucose, maltose, cellobiose, and melibiose (6). A lactose dehydrogenase has been isolated from *Pseudomonas graveolens* which oxidizes lactose to lactobionic acid; D-glucose, D-galactose, D-mannose, D-talose, D-ribose, D-xylose, L-arabinose, maltose, and cellobiose also serve as substrates (30).

The direct oxidation of disaccharides, as suggested here for maltose and cellobiose, has, to our knowledge, not been described previously in mammalian tissues. The reaction occurring with these two disaccharides cannot be explained by incidental hydrolysis of the disaccharides, since the constituent monosaccharide, glucose, is not a substrate for the enzyme. The NAD-dependent oxidation of some ketohexoses and heptuloses was another surprising finding since such reactions are not presently recognized. Isomerization of the keto sugar to the aldo sugar with subsequent oxidation of the latter appears to be a reasonable explanation. It is possible that this isomerization-dehydrogenation process is catalyzed by the same enzyme as described for liver alcohol dehydrogenase (20, 21). However, it is possible that a different type of oxidation reaction is occurring with the keto sugars. Recently a non-nucleotide-dependent direct oxidation of D-fructose to 5-keto-D-fructose has been detected in certain bacteria (6, 31–33).

Although no direct evidence has been obtained to establish whether the product of sugar oxidation is a $\gamma$- or $\delta$-lactone, it is probably a $\delta$-lactone in the case of the two disaccharides since the reducing monosaccharides exist in the pyranose form and the $\gamma$-carbon is engaged in the glucosidic linkage.

In rat liver, the activities of galactose dehydrogenase and galactokinase (34) are of similar magnitude (5 to 10 m$\mu$moles per min per mg of protein) when each is assayed near its optimum conditions. How this relates to their relative activities *in vivo* is presently uncertain. The galactose $K_m$ of the dehydrogenase enzyme, $2.6 \times 10^{-2}$ M, is greater than that of galactokinase, $1.5 \times 10^{-4}$ M (34); but in crude preparations the galactose $V_{max}$ of galactose dehydrogenase (5.7 m$\mu$moles per min per mg of protein) is greater than that of galactokinase (1.2 m$\mu$moles per min per mg of protein). Furthermore, galactokinase activity is inhibited by galactose (detectable at 3.2 mM) as well as by the product, galactose-1-P (60% inhibition with 1 mM for the enzyme of newborn animals, 50% inhibition with 5 mM for the enzyme of adult animals) (34). It is possible that in certain situations, such as during high galactose feedings or during infancy, the galactose dehydrogenase enzyme plays a more significant role.

The findings presented here indicate that the liver of young rats has greater capacity than the adult liver for direct oxidation of galactose at C-1. The changes in rat liver galactose dehydrogenase activity occurring with advancing age are very similar to those observed with galactokinase (34) and galactose-1-P uridyltransferase (35), and all parallel closely the changes in over-all metabolism *in vitro* of galactose by rat liver as measured by rate of uptake from the medium and oxidation to $CO_2$ (36). The high activity of these liver enzymes in young animals coincides with the time of high dietary galactose consumption and may be important in preventing tissue accumulation of galactose-1-P, which is thought to be a toxic substance.

Patients with congenital galactosemia, who lack galactose-1-P uridyltransferase, can nevertheless oxidize galactose well, and in certain cases the oxidation of galactose-1-$^{14}$C to expired $^{14}CO_2$ is nearly normal (37, 38). Only two patients have had liver biopsies for study of conversion *in vitro* of galactose-1-$^{14}$C to $^{14}CO_2$, and in both the rate was nearly normal (39, 40). It is possible that galactose dehydrogenase and its subsequent steps may account for the residual galactose metabolism in galactosemic individuals. The fact that galactosemia is frequently diagnosed by the inability of red or white blood cells to convert galactose-1-$^{14}$C to $^{14}CO_2$ is also consistent with the virtual absence of galactose dehydrogenase in red or white blood cells (1). UDP-galactose pyrophosphorylase has been suggested as a possible bypass for the defective enzymatic step (41, 42). However, this enzyme in crude liver supernatant fractions is at least 4000 times less active than galactose dehydrogenase (0.0025 m$\mu$mole per min per mg of protein at 38° compared to 7 m$\mu$moles per min per mg of protein at 20°).

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